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Character of Agent Inducing Leukemia in Newborn Mice.* (22697)

JACOB FURTH, RITA F. BUFFETT, MARIA BANASIEWICZ-RODRIGUEZ,† AND ARTHUR C. UPTON

Children's Cancer Research Foundation, Children's Medical Center and Department of Pathology, Harvard Medical School, Boston, Mass., and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

It is now well established that there are filterable agents capable of causing leukemia or tumors, or both, in several species of animals, and Stanley(1) concluded that many, if not all, tumors in man as well as in animals could be caused by viruses. Of current interest is the virus‡(2) in tissues of high leukemia strains of mice that will induce leukemia when injected into mice of low-leukemia strains less than 1 day old. Technical difficulties have greatly handicapped confirmation and research on the nature of this agent: the necessity of using animals less than 12 hours of age; the extraordinary host specificity; the long latency period (about 7-12 months) which is close to that of spontaneous leukemias; the variable and usually small percentage of leukemias induced; the necessity of using large numbers of controls to ascertain that the tumors were induced and not spontaneous; and the performance of bioassays to further exclude contamination of the inoculum with living cells.

Two sets of experiments are reported here. The first was initiated with Dr. Ludwik Gross. Motivated by the thought that if a virus causes leukemia it may play a determining role in leukemia induction by radiation, Dr. Gross was invited to Oak Ridge in July, 1953, to demonstrate his technic. The second set of experiments were set up in Boston primarily to establish the sensitivity of different low-leukemia strains to the agent in Ak tissue. The immunogenetic character of many leukemias was tested by transplantation assays, since one or a few immature or leukemic cells from Ak donors may have been present in the inoculum and may have survived when grafted in newborn mice, giving rise to leukemia close to the "leukemia age." It was assumed that virus-induced leukemias have the immunogenetic character of the recipient strain, and cell-induced leukemias that of the donor strain.

Materials and methods. Strains of mice. Low-leukemia strains, C3H (abbr. H), LAF_1, DBA/1, BALB/c, Rf, and C57BR, were used. The tissue extracts containing the agent were prepared from Ak leukemic mice. Sublines of Ak and H mice are indicated as follows: a = Andervont, g = Gross, r = Russell, b = Bittner, R = Rockefeller Institute (Lynch), o = Oak Ridge, eb = mam-

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‡ The terms virus and agent are used interchangeably for the substance in tissue extracts capable of inducing or hastening the development of leukemia.

TABLE I. Results of Injections of Ak Leukemia Extracts into Newborn Mice.

Donor		Recipients					
Strain	Material	Ha	Hg	Hr	Hb/Hr	Total No.	Total %
Ak/g	Centrifugate	2/5*	—	3/25	—	5/ 30	16.7
"	Filtrate	4/16	3/4	1/34	2/11	10/ 65	15.4
Ak/o	"	1/2	—	1/17	5/19	7/ 38	18.4
AkR	Centrifugate	—	—	4/6	—	4/ 6	66.7
	Total No.	7/23	3/4	9/82	7/30	26/139	
	%	34.8	75.0	11.0	23.3		18.6
	Control No.	1/10	0/15	4/45	7/34	12/104	
	%	10	0	8.8	20.6		6.7

* No. positives (leukemias) over total No. of mice inj.

mary agent-free line of the Roscoe B. Jackson Memorial Laboratory. The use of the indicated sublines of C3H and Ak as both source of the agent and host was by necessity. *Preparation of extracts.* The technic of Gross was slightly modified in the second experiment. Extracts of leukemic tissue were prepared from a recently isolated strain of transplantable AkR leukemia. Spleen, lymph nodes (not including cervical nodes in the second experiment because of their proximity to the salivary gland which may be carrier of another virus), thymus, and a small piece of liver were ground in a mortar with enough cold normal saline to make a 20% suspension, centrifuged at 3000 rpm for 10-15 minutes in a refrigerated centrifuge set at 0°C, and the supernatant fluid drawn off. The supernatant fluid was recentrifuged at 0°C at 3000 rpm for 15-30 minutes or 7000 g for 10 minutes, and the supernatant fluid again drawn off. In the second experiment, the floating fat was removed with a sterile swab. The extract was kept on ice in the refrigerator and used within 24-30 hours after preparation. Filtration was omitted in the second set of experiments in order to avoid further weakening of the extracts and because emphasis was placed on the character of induced leukemic cells. Instruments and equipment were chilled. *Inoculations.* Babies less than 12 hours old, and some 12-24 hours old were injected subcutaneously in the back with 0.05-0.1 cc of inoculum through a 27-gauge needle. In some instances, supplementary intraperitoneal injections were made to make a total dose of 0.1 cc. Implants of leukemic cell suspension in adults were made intramuscularly in the thigh. *Termination of experiments.* In the first experiment, nearly all mice were observed until natural death. The second experiment was terminated when the last surviving animals were 10-15 months of age (about 38% of the total number being younger than one year). This seemed permissible because of our limited objectives, the spontaneous leukemias in question being predominantly thymic and appearing mostly under 10 months of age.

Results. First experiment. The results of the first set of injections of Ak leukemia extracts into newborn mice (all less than 12 hours old) are summarized in Table I. In one subline (Hb/Hr), the incidence of leukemia among the controls was almost as high as in the experimental group and higher than the average in the various H sublines used. In subline Hr the difference between experimental and control groups was also slight. Transplantation assays were made on available mice in ignorance of the marked subline differences among C3H (=H) mice (Table II). On first glance, the results appear to

TABLE II. Transplantation Assays of 4 Leukemias Developing in Agent Injected Mice.

Donor			Recipient*		
No.	Strain	Material	AkR	Ak/o	Hr
Hb/Hr 26	Ak/o	Filtrate		0/ 6	0/11
10	Ak/g	"		0/ 4	1/ 5
Hr 47	AkR	Centrifugate	0/20		12/12
45	"	"		0/ 4	1/ 6
		Total	0/20	0/14	14/34

* Each recipient received intraper. 0.1 ml of a leukemic cell suspension, and was observed until natural death or for a period of at least 3 mo after inoculation.

TABLE III. Induction of Leukemia in Newborn Mice with AkR Leukemic Tissue Extract.

Strain	Material†	No. survivors 4 mo*	Leukemia		Months postinjection							
			No.	%	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–15
LAF_1	E	53	0									
	S	66	0									
Heb	E	54	8	15	3	1		1	2		1	
	S	42	0									
DBA/1	E	47	9	19		1	1	1	1	1		4
	S	35	0									
BALB/c	E	33	1	3			1					
	S	33	0									
Rf	E	29	10	38	1	2	2	2	1		2	1
	S	43	0									
C57BR	E	55	1	2							1	
	S	53	0									
AkR	E	34	26	76	2	3	5	6	3	3	3	1
	S	21	10	48			1	1	1	4	2	1

* Discounting from total a few mice on which adequate autopsy could not be performed.
† E = Extract; S = Saline.

confirm the findings of Gross in that 3 of 4 leukemias could be grafted on H mice, none on Ak mice, and one on neither strain. However, only one (Hr47) had a distinctly H (Hr) type of leukemia, but in this subline the difference in incidence of leukemia between agent-injected and control mice was negligible; hence the disease might have been spontaneous. The two leukemias arising in the Hb/Hr subline, which one would expect to be transplantable to all Hr mice, took in only 1 of 16 animals. Thus these findings cannot be taken as evidence that these leukemias were of the recipient immunogenetic types and hence induced by a virus. Interpretation of these results calls for ascertaining the degree of homozygosity and the immunogenetic relationship of the various sublines.

Second experiment. Leukemia induction. It seemed desirable to search for a low-leukemia strain with high sensitivity to the Ak agent, preferably a strain with less immunogenetic relationship with Ak as is known to exist between some sublines of C3H and Ak (3,4). Accordingly, newborn mice of 7 strains were injected with tissue extracts from mice with AkR leukemias (Table III). The extracts were presumably cell-free, and transplantation assays (Table V) suggest that most induced leukemias were certainly not induced by contaminating cells.

Four strains of animals proved fairly sensitive to induction of leukemia with Ak agent: Heb, DBA/1, Rf, and the high-leukemia strain, AkR, from which the agent was obtained (Table III). Leukemia was induced in 15% of a breast tumor agent-free subline of C3H (Heb) mice; none occurred among the controls. (Sublines of greater sensitivity were not available.) Leukemia was induced in 19% of DBA/1 mice; none occurred among the controls.

Of the low-leukemia strains, Rf proved most susceptible; 38% of agent-injected mice developed leukemia, at a time when none of the controls had it. Originally, the Rf strain had only 2-3% spontaneous leukemias, and it had no genetic relation to the Ak strain (3). In recent years, a small percentage of Rf mice "take" Ak leukemic cells. The spon-

TABLE IV. Transplantation Range of AkR Leukemic Cells.

Recipient strain	Leukemia	
	Adults	<1 day old
LAF_1	0/ 11	4/ 5
Heb	0/ 27	
DBA/1	0/ 7	
BALB/c	0/ 11	
Rf	17/174	
C57BR	0/ 8	12/13
AkR	18/ 19	10/10

One spontaneous leukemia in an Rf mouse was grafted on Rf and AkR mice with results as follows: Rf hosts 6/6, AkR hosts 0/5.

TABLE V. Transplantation Assay of Leukemias of Mice Injected with AkR Extracts.

Leukemia donor No.	Age†	Original passage: In strain of origin Result	Original passage: In strain of origin Latency‡	Original passage: In AkR Result	Original passage: In AkR Latency	Subpassage I: In strain of origin Result	Subpassage I: In strain of origin Latency	Subpassage I: In AkR Result	Subpassage I: In AkR Latency	Subpassage II: In strain of origin Result	Subpassage II: In strain of origin Latency	Subpassage II: In AkR Result	Subpassage II: In AkR Latency
Heb 17	141	*2/6	55, 93	1/6	97	7/11	29–37						
						3/ 6	36	*1/6	83	0/5		5/5	44–48
20	141	*4/4	21–45	1/6	84	17/17	16–21	*6/6	23–28	4/4	15–37	4/4	14–15
						19/19§	19–20						
16	251	0/5		2/5	60, 205								
Rf 55	163	0/3		4/5	92–183								
56	163	3/5	16–18	1/5	69								
57	262	5/5	18–60	2/6	59, 108								
7	217	*4/6	27–42			1/ 4	44	0/4					
				*3/6	83–100			3/3	29–33				
12	143	2/6	136, 159	1/5	137								
16	237	0/6		0/5									
DBA/1 1	435	2/5	53, 82	0/3									
8	428	1/5	74	0/4									
9	428	*5/5	45–49	0/4		5/ 5	18–20	0/5					
BALB/c 13	186	*9/9	17–19	0/8		1/ 3	48	0/4					

* Indicates group from which subpassage was made. † In days, when mouse was sacrificed for transplantation. ‡ No. of days after graft.
All AkR recipients were 1½-2½ mo of age when grafted. § Frozen cells from same donor as transfer above.

TABLE VI. Mortality Data on Newborn Mice Injected with Leukemia Extracts (Second Experiment).

Strain	Material	No. inj.	No. weaned	% loss	No. survivors 4 mo	Mortality: 1–4 mo No.	Mortality: 1–4 mo %	Mortality: 4–10 mo 4–6	Mortality: 4–10 mo 6–8	Mortality: 4–10 mo 8–10	Total mortality, 4-10 mo: Leukemia No.	Leukemia %	Other causes No.	Other causes %	Combined No.	Combined %
LAF_1	E*	87	59	32	54	5	8†	1	0	0	0		1	2‡	1	2‡
	S*	110	66	40	66	0		0	0	0	0		0		0	
Heb	E	112	62	45	55	7	11	9	9	6	7	13‡	17	31	24	44
	S	100	51	49	43	8	16	2	2	0	0		4	9	4	9
DBA/1	E	155	55	65	50	5	9	4	7	6	5	10	12	24	17	34
	S	138	38	71	38	0		5	0	3	0		8	21	8	21
BALB/c	E	54	39	28	34	6	13	2	6	1	1	3	8	23	9	26
	S	52	39	25	33	6	15	1	3	2	0		6	18	6	18
Rf	E	148	42	72	29	13	31	4	6	2	8	27	4	14	12	41
	S	112	49	56	43	6	12	0	0	0	0		0		0	
C57BR	E	82	60	27	55	5	8	2	1	1	0		4	7	4	7
	S	77	55	29	53	2	4	4	1	2	0		7	13	7	13
AkR	E	103	63	61	36	4	10	6	12	6	22	61	2	6	24	67
	S	103	77	75	22	4	15	2	3	5	7	32	3	14	10	46

* E = Extract; S = Saline. † % of those weaned. ‡ % of those surviving 4 mo.

taneous incidence of leukemia remained low in the Rf strain, the unique feature of which is an extraordinary susceptibility to the induction of myeloid leukemia by ionizing irradiation(5,6).

Injection of newborn AkR mice with AkR extract greatly shortened the latency period of leukemia and increased the leukemia incidence as reported recently by Rudall *et al.* (7).

Immunogenetic Relationships. Table IV shows that adult Rf mice are sensitive to AkR cells, but "takes" occur in only about 10% of them when injected with large numbers of Ak leukemic cells. Adult mice of the other 5 strains proved resistant to AkR cells. Newborn mice of 2 strains (LAF_1 and C57BR), which proved resistant to the agent, were receptive to AkR cells. This indicates that histoincompatibility forces do not function in newborn mice.

Transplantation assays for the immunogenetic character of 13 leukemias developing in agent-injected mice (3 in Heb, 6 in Rf, 3 in DBA/1, and 1 in BALB/c) are summarized in Table V. All leukemias were grafted on mice of the strain of origin and on AkR mice. In 5 cases, further subpassages were made.

In the Heb strain, the 3 leukemic mice were 141-251 days old when sacrificed for transplantation assay. Two had marked generalized leukemia with thymic involvement, and proved to be immunogenetically related to the AkR strain. The grafted leukemias grew faster and in greater percentage in the strain of origin than in AkR. However, when a subpassage was made from the first-generation graft in an AkR mouse from Heb 17, leukemia developed in all of 5 AkR and in none of the strain of origin (Table V). It is unlikely that the leukemia was spontaneous in this donor AkR mouse because the latter was only 4 months old when sacrificed. The leukemia in Heb 20 certainly appeared "bivalent," since it "took" in all injected AkR and Heb mice. The third, presumably agent-induced leukemia (Heb 16), was microscopically an early case. Transplantation failed in the strain of origin but apparently succeeded in 2 AkR mice. One of these was probably transplanted, since it developed in a 4-month-old mouse (60 days after the graft) and was nonthymic. The second, being thymic and occurring late, may have been spontaneous.

Of the 6 transplanted leukemias arising in Rf mice, one grew in AkR only, 4 grew in both Rf and Ak, and one in neither strain. The one which appeared to be of the AkR type (Rf 55) could have been derived from Ak cells contaminating the extract. If so, it was latent in the Rf host for about 163 days. In general, the leukemias that were "bivalent" grew faster and in greater percentage in Rf than in AkR mice. Again, a subpassage from an AkR leukemia grew well in AkR. It is possible that this was spontaneous, since the donor mouse was nearly 5 months old when this subpassage was made. All 4 Rf mice grafted from this AkR died of infection 17 days after the graft, and had to be discounted. The 3 DBA/1 and 1 BALB/c leukemias grew only in the strain of origin. The very long induction period (428-435 days) of the 3 DBA/1 leukemias raises the question whether these cases were not spontaneous.

These data point to the necessity of carrying out transplantation assays on a much larger scale and with the use of proven highly inbred strains of mice with good genetic markers.

Mortality in agent-injected mice. Duran-Reynals has shown(8) that an oncogenic virus can cause a non-neoplastic disease in newborn animals. Mortality data (Table VI) were gathered to answer the question whether or not diseases other than leukemia might have been caused by the agent and whether some leukemias might have been missed at autopsy.

Table VI indicates that, in general, infant mortality was equally high among agent- and saline-injected animals. Indeed, in 5 strains mortality was slightly higher among the controls and in only 1 (Rf) was it distinctly higher among the extract-injected animals.

Leukemias did not occur until after the mice were 4 months old. In the interval between weaning and 4 months of age, mortality was higher among agent-injected mice in 4

strains, but in only 2 of these (DBA/1 and Rf) was there a definite increase of leukemia incidence in subsequent months. In 2 strains in which leukemia development was hastened and/or increased (AkR and Heb) mortality was higher among the saline-injected animals, even during the first 4 months. Mortality was caused by common infections and parasitisms.

In the 4 low-leukemia strains in which leukemia developed in extract-injected animals and was absent in saline-injected animals during the first 10 months of life, mortality from causes other than leukemia was invariably higher among the agent-injected animals, the difference being greatest in the 2 strains in which the incidence of leukemia was the highest (Rf and Heb). Since no systematic microscopic examination was made in animals grossly free of leukemia, it is not possible to state whether all those mice recorded to have died from causes other than leukemia were actually free from this disease, or whether the presence of the agent had lowered resistance to common infections, or otherwise caused death. The total mortality between the ages of 4 and 10 months appears to be as good an index of viral activity as the presence of grossly detectable leukemia.

Salivary gland and other tumors. Gross suggested that the virus carried in Ak mice is multipotent, capable of producing salivary gland and other tumors, or that Ak mice carry several oncogenic viruses. At autopsy, careful search failed to reveal neoplasms other than leukemias, except for two questionable salivary gland tumors in the first series. These findings do not support the view that the salivary gland tumors are caused by the same agent that induces leukemia.

Three salivary gland tumor strains (C3H) received from Dr. Gross were transplantable in C3H mice with variable success; none took in 100% of the animals and some regressed. They were not transplantable in Ak mice. This is in line with the work of others(9;10), indicating poor transplantability of these parotid tumors. The parotid glands of many C3H mice carrying grafted salivary gland tumors were examined microscopically. In none were incipient tumors noted, nor was there any evidence of other tumors or leukemias in hosts bearing such grafts.

Discussion. Three processes will now be considered by which leukemia can be caused: transplantation, induction, and transduction. Cellular grafts from spontaneous leukemias retain, as a rule, the genetic character of donor cells, whereas grafts from induced leukemias resemble closely or are identical with those of the host cells. Although this is the rule, it has also been reported that methylcholanthrene-produced leukemias differ among themselves immunogenetically(11). Bioassays indicate that leukemic cells are not present in young animals with genetic liability to leukemia(12). A primitive hemopoietic cell may become established in homologous newborn mice of diverse strains, and undergo leukemic transformation after a latency period (close to that of spontaneous leukemia). In this case, the leukemic cells are also expected to be of the donor type, and the process would be that of *transplantation* of precursor cells. Gross(13), and Woolley(14) working closely with Gross, have stated that their virus-induced leukemias were of the recipient type; this would indicate *induction.* Analysis of our transplantation results points to difficulties of interpretation, and suggests the possibility that some induced leukemias have the genetic character of both donor and recipient. Such a finding would be best explained by supposing that the transmitting agent is related to chromosomal matter (probably nucleoprotein) of the leukemic cells or their precursors which, like the DNA of certain microorganisms, can enter the reproductive matter (DNA genome) of other "infected" cells, either multiplying with them or causing perpetuation of their special genic characteristic by some other mechanism. Lederberg(15) named such processes *transduction.* Although it is doubtful that the transformations induced with lysogenic viruses (bacteriophage) are analogous with that of induced transformation of pneumococci, the term of Lederberg is accepted as a matter of convenience. In contrast, common infectious

viruses of mammals are best conceived as fully perpetuating themselves, the host merely contributing a biochemical environment, favorable to their multiplication.

The experiments here reported are inadequate to arrive at a definite conclusion on the nature of the agent which induces or hastens the development of leukemia in mice. Information is wanted on the effects of injection of extracts from low-leukemia strain mice,§ characterization of agents after passage through different strains, better correlation of immunologic and genetic relationships, and attempted inactivation of the agent with DNA-ase. Simple technical difficulties as encountered here in interpreting findings with this type of experiment will have to be overcome. How to certify inocula to be free from cells, and strains for adequate homozygosity? How long is the latency period of leukemias caused by transplantation of one or a few leukemic cells shortly after their origin? It is well known that neoplastic cells rapidly gain growth vigor in the course of passages, but it is not known when the neoplastic transformation occurs and how rapidly leukemic cells multiply in the original host. Inadequate inbreeding of the mouse strains, or lack of complete autonomy or genetic difference between strain and induced neoplastic cells could possibly account for non- or poor transplantability of some leukemias in the primary transfers. The strains used by us were adequately inbred for uniform success in transplantation of well-established leukemias, but inbreeding is a relative matter. Subtle immunogenetic changes are conceivable even in the course of neoplastic transformation in spontaneous leukemias, and antigenic simplification is a common occurrence in the course of transplantation.

A major problem requiring clarification is the type of leukemia induced in relation to the source of the agent. In the Ak strain, most spontaneous leukemias (80-90%) are thymic in origin. Is the thymus the sole or most sensitive organ attacked by this agent? In the present study all leukemias in agent-injected Ak mice were lymphoid with thymic involvement. In the other strains, however, many were generalized with involuted thymuses. Typing of leukemias requires special efforts not done systematically in the present study. The morphologic problems to be resolved include those of differentiation of advanced myeloid leukemoid reaction from leukemias, and of granulomatous lesions from neoplasms of monocytes (reticulum cells). Such a study calls for correlation of morphology with bioassays for malignant (autonomous) cells. Spontaneous leukemias in Ak mice are preventable by thymectomy(3). It follows that leukemia induction by this Ak agent would also be preventable by thymectomy. Attempts to recover the agent from nonthymic, spontaneous, and variously induced leukemias, and determination of the range of agents from different sources will have to be done. Since most nonthymic leukemias appear late in life, lifespan studies will be unavoidable. The Rf strain appears well suited for such studies because of its broader spectrum of sensitivity. In this strain, ionizing radiation causes thymic lymphomas (preventable by thymectomy)(5), myeloid (not so preventable), and reticulum-celled (monocytic) leukemias.

Three processes have been mentioned that could explain the development of leukemias following injection of tissue extracts from high-leukemia strain mice: transplantation, induction, and transduction. The first can now be excluded as a mere occasional complicating factor, the latter two are possibilities that merit equal consideration. Much has been said by others in favor of induction; the present work underlines the theory of transformation (transduction). Experiments suggesting it have been described and contradicted(3,16), and those here reported are likewise not conclusive. But the theory of transduction based on experiments on pneumococcal transformation would explain best the observed facts: the necessity of using newborn mice; the reported high sensitivity of strains which have some genetic relation to the Ak strain; the non-infectiousness of this

§ Latarjet (personal communication) failed to demonstrate an agent in his low-leukemia line 17.

agent under natural conditions; and the very long latency period, which is about that of the spontaneous disease. Latarjet(17) considered the possibility of the agent being similar to a prophage, but his experiments argue against this.

In general, causation of leukemia by virus can be conceived as: a) Stimulation of normal cells by intracellular specific or nonspecific "parasites." This would imply that the leukemias are virus-conditioned neoplasms. b) Induction of somatic mutation by a resident virus, proposed by Murphy(18) who named avian tumor viruses "mutagens." Mutation and abnormal differentiation, another possibility, imply an irreversible induction change in the cells, mutation being genetic and differentiation nongenetic in character. c) Recombination of chromosomes as occurs with transduction of genetic features of pneumococci and other microorganisms. Presently, all these possibilities(16) are useful as "working hypotheses."

Summary. 1. Tissue extracts from mice of the high-leukemia strain Ak injected into newborn mice greatly increased the leukemia incidence in Rf, DBA/1 and C_3Heb mice, and greatly hastened the development of leukemia in AkR mice. 2. Mortality without gross anatomic manifestation of leukemia was also enhanced in extract-injected mice of low-leukemia strains. 3. Although all leukemias in Ak mice were lymphoid with thymic involvement, many leukemias in the low-leukemia strains were of non-thymic types. 4. Transplantation assays of leukemias induced in the low-leukemia strains disclosed a heterogeneity in types, some being related immunogenetically with both host and donor strain. 5. Neither salivary gland nor other tumors were induced by the leukemia agent studied. 6. The available information suggests consideration of a process analogous with DNA-induced transformation (transduction) of genetic properties of microorganisms rather than to a natural type of viral infection.

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Oxygen Consumption in the Rat During Prolonged, Acute Starvation. (22698)

R. G. Sisson and S. Lang (Introduced by M. D. Pareira)

Surgical Research Laboratories of the Jewish Hospital of St. Louis, Mo.

The oxygen consumption of animals(1,2) and humans(1,2,3) is known to be depressed by starvation. There is also ample evidence that atrophic changes occur in the thyroid gland(1,4) and that histological and histochemical alterations appear in the anterior pituitary gland during inanition(1,4). These changes are assumed to be the explanation for the depression in oxygen consumption during starvation. It is, however, entirely probable that such changes are secondary to alterations of peripheral cellular metabolism.

The latter hypothesis was tested in hypothyroid, euthyroid and hyperthyroid rats subjected to prolonged, acute starvation.

Methods and materials. All animals were young, mature female rats of the Holtzman Farm weighing between 200-250 g. Thyroidectomy was performed under sodium pentabarbital (nembutal, 30 mg/kg I.P.). The soft tissues of the necks of random experimental animals were removed *en bloc,* sectioned serially and examined histologically for evaluation of completeness of surgical ablation of the gland. Animals were used 5 to 6 weeks after thyroidectomy. Hyperthyroid animals were prepared by subcutaneous, daily injection of 10 γ of 3-5-3′ triiodothyronine.* Injections were begun 3 days prior to the commencement of starvation. Starvation was instituted by removal of food from the cages at 9:00 a. m. of the day on which the experiment was begun. At noon, the rats were weighed on a triple beam balance to the nearest 0.5 g, which was taken as the initial weight. Animals were weighed approximately the same time each day thereafter. After removal of food (Purina Laboratory Chow) the animals received water *ad libitum* but no food. All animals were housed in individual cages and kept in an airconditioned room (about 75-80°F.) Oxygen consumption was measured by a method described by Grad(5). At least three 15-minute determinations were averaged to establish each oxygen consumption point reported. The formula used to express the oxygen consumption is: Liters/m^2/hr $= \frac{11.2 \times (wt^{2/3})}{100}$ where weight is in kg and the figures corrected to standard temperature only.

TABLE I. Oxygen Consumption in the Normal, Thyroidectomized and Triiodothyronine Treated Rat.

Treatment	No. of animals	O_2*	S.D.†
Normal	52	6.63	.43
Thyroidectomized	6	5.20	.51
Triiodothyronine	11	8.10	.86

* O_2 = Oxygen consumption in liters/m^2/hr.
† S.D. = Stand. dev. of mean.
Triiodothyronine was given for 3 days (10 γ/day subcut.) and oxygen consumption measured on 4th day. Animals were without food for 4 hr.

Results. Determinations of oxygen consumption in a series of 52 normal animals resulted in a mean value of 6.63 $\pm$ 0.43 liter/m^2/hr. A comparison of the oxygen consumption of normal, thyroidectomized and triiodothyronine treated rats on day zero (starting day of starvation) is shown in Table I.

Fig. 1 shows the daily oxygen consumption course of the starving rats in each treatment group. Because of space and time limitations it was impossible to measure each animal's oxygen consumption daily. Table II indicates number of animals used in determining each point of the graph. There was no selection of animals to be tested on any given day. No observation has been deleted from this report. The original size of the groups in the experiment was: 11 intact, 11 triiodothyronine treated animals and 12 thyroidectomized animals.

* We are indebted to Dr. T. A. Schwab of Smith, Kline and French Laboratories for a generous supply of triiodothyronine.

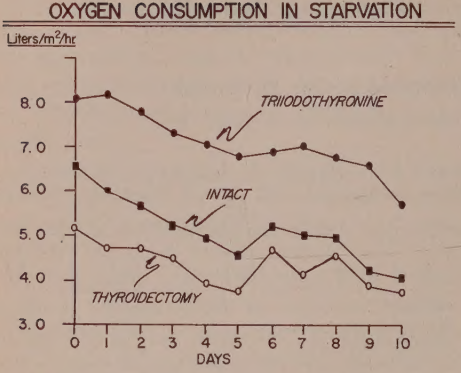


FIG. 1. Oxygen consumption during starvation of female rats. Triiodothyronine was given subcut. each day in the amount of 10 μg and was started 3 days prior to the commencement of starvation. Thyroidectomized animals were prepared surgically and allowed 5-6 wk to develop hypothyroidism.

It will be noted that there is a decrease in oxygen consumption in all 3 groups subjected to progressive starvation. The decrement seems absolutely greater in the normal than in the triiodothyronine treated or in the thyroidectomized groups. The thyroidectomized group shows the least decline in oxygen consumption during acute starvation. In the triiodothyronine treated and thyroidectomized groups there seems to be a relative plateau reached at about the 4th day and maintained until about the 9th day. The triiodothyronine group showed a considerable mortality by this time (commencing on the 6th day of starvation), and the experiment was terminated on the 10th day of starvation (which is about 0.5 day longer than the mean survival time for intact starving rats in our laboratory; range 9 to 11.5 days). The thyroidectomized animals live 2 to 2½ times as long as the intact, starving animals.

There seems to be a marked similarity between the triiodothyronine treated and thyroidectomized curves, while the curve of the intact starving rats deviates somewhat from their general configuration by a more sustained and steeper oxygen consumption fall.

TABLE II. Number of Animals Determining Each Point on Graphs of Fig. 1.

Treatment	Day 0	1	2	3	4	5	6	7	8	9	10
Triiodothyronine	11	10	9	10	10	2	2	8	8	7	2
Intact	52	10	5	9	8	2	3	5	8	4	3
Thyroidectomized	6	5	8	3	5	2	1	5	5	3	3

Discussion. It is demonstrated that starvation is capable of reducing the oxygen consumption of the female rat independently of thyroid activity. This decrement in oxygen consumption is apparent in rats in which the thyroid gland is absent, as well as in animals rendered hyperthyroid by administration of 10 gamma of triiodothyronine per day.

It should be pointed out that administration of a constant daily amount of triiodothyronine to the starving rat amounts to an increasing daily dose when calculated on the basis of dose to body weight.

Whether this depression in oxygen consumption is due to loss of specific dynamic action of food, lack of sufficient available substrate at the cellular level, or other causes, can not be answered by the present experiment. Investigation of these various possibilities is in process.

Summary. Hypothyroid, hyperthyroid and euthyroid rats were subjected to acute starvation to extremis. Their oxygen consumption was measured daily. All groups showed an approximately equivalent early depression of oxygen consumption which is independent of the absolute level of the initial oxygen consumption.

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Effects of *trans* Fatty Acid Isomers upon Essential Fatty Acid Deficiency in Rats.* (22699)

Ralph T. Holman and Erik Aaes-Jørgensen

Department of Physiological Chemistry, and the Hormel Institute, University of Minnesota, Austin, and Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark

Recently it has been found that diets containing hydrogenated fats cause degeneration of spermatogenic tissue in weanling rats(1). Hydrogenated fats contain considerable proportions of *trans* isomers of the unsaturated fatty acids(2). Therefore it became of interest to learn if known *trans* isomers of the unsaturated acids have any effect upon spermatogenic tissue and if they are deposited in the animals. The most obvious unnatural fatty acid isomers to test for this effect are the *cis, trans* and *trans, trans* isomers of linoleic acid. These acids have been demonstrated to possess no essential fatty acid activity(3,4) and they are not oxidized by lipoxidase. Moreover, the all *trans* isomer of linolenic acid has been found to act as an effective competitive inhibitor of lipoxidase oxidation of linoleate(5), and it might be expected that the *trans* isomers act as competitive inhibitors of enzyme reactions involving essential fatty acids. Elaidic acid might also be suspected of antagonism to the essential fatty acids by the same reasoning. Therefore it was decided to test these substances for their overall effects on essential fatty acid (EFA) deficiency and upon the development of spermatogenic tissue. The quantities of these acids available did not allow a detailed or full investigation of their effects, but some effects have been observed in a few animals.

Methods. Male 15-day-old rats were fed a depletion diet containing 18% casein, 67% sucrose, 4% Wesson salts, 4% celluflour, 1% hydrogenated coconut oil, 1% cholesterol, 2% sulfasuxidine, and 3% urea plus required vitamins. The inclusion of cholesterol has been demonstrated to accelerate EFA deficiency(6). At the end of 3.5 months, all rats had developed severe EFA deficiency symptoms. The rats were then divided into pairs and were given a diet containing 18% casein, 73% sucrose, 4% salts, 4% celluflour, and 1% hydrogenated coconut oil plus the required vitamins. The fatty acid esters were then administered as oral supplements at the rate of 0.1 ml per day for 3 weeks. The rats were weighed and scored biweekly during the period of supplementation. At the end of this period, they were killed, and the left testis was removed from each for histological examination. The carcasses were extracted with boiling ethanol and petroleum ether. The lipid was saponified, the unsaponifiable matter was removed, and the free fatty acids recovered. The fatty acids from the odd-numbered rats were separated into "saturated" and "unsaturated" acids by crystallization from 10 volumes of petroleum ether per weight of acids at —40°. Fatty acids from even numbered rats were esterified with methanol for use in determining the content of *trans* unsaturation. The values are expressed as elaidic acid. Ethyl elaidate (*trans*-9-octadecenoic acid ethyl ester) was prepared from elaidic acid (m.p. 44°). Ultraviolet absorption was $k_{268} = 0.025$ and $k_{233} = 0.18$. Ethyl linolelaidate (*trans*-9, *trans*-12-octadecadienoic acid ethyl ester) was prepared by the method of Kass and Burr(7). Infrared spectrum showed no *cis* double bonds to be present. Ultraviolet absorption indicated the presence of about 7% conjugated diene acids. $k_{268} = 0.18$, $k_{233} = 6.7$. Methyl *cis*-9, *trans*-12-linoleate was prepared by Dr. O. S. Privett. The preparation had strong absorption bands at 10.28 μ (*trans*) and 3.3 μ (*cis*). $k_{233} = 0.52$ and $k_{268} = 0.19$. Ethyl linoleate (nat. *cis*-9, *cis*-12-octadecadienoic acid ester) was prepared by low temperature crystallization of safflower oil fatty acids. Infrared spectrum showed evidence of a trace of *trans*

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TABLE I. Effects of Fatty Acid Supplements upon E.F.A.-Deficient Rats.

Rat No.	Supplement	Wt gain, g	Score change*	Testis /body wt, %	Spermatogenic tissue†	Carcass fatty acids, g	*Trans* acids, %‡	Total *trans* acids, g‡	Gain/g *trans* acid‡
1	Ethyl elaidate	48	2.0		1	12.8			
2		40	− .5		2	12.2	1.4	.17	258
3	" linolelaidate	27	2.0	.13	1	20.8			
4		10	2.0	.22	1	13.5	2.7	.36	50
5	Linolelaidate +	71	0	.28	3	18+			
6	linseed oil	74	−1.5	.30	3	29.6	3.9	1.15	63
7	*cis,trans* linoleate	21	2.0	.34	3	12.6			
8		9	3.0	.20	2	9.8	3.0	.29	51
9	*Idem* + linseed oil	51	− .5	.26	4	32.3			
10		63	−2.0	.18	2	25.5	2.3	.59	97
11	Linolenate	42	− .5	.27	2	28.1			
12		92	1.5	.29	2	28.7	.9	.26	259
13	Linoleate	56	−2.5	.24	2	27.1			
14		50	−4.5	.31	4	33.5	1.2	.40	132
15	Linseed oil	62	−2	.35	4	22.7			
16		61	1.5	.28	4	29.3	0	0	0
17	None (fat-free)	31	1.0	.29	2	11.5			
18		36	.5	.22	1	19.0	0	0	0
19	Deficient initial					4.0			
20	controls					6.1	1.4	.1	

* Scores of 0-3 for scaliness of feet, 0-4 for scaliness of tail, and 0-2 for roughness of hair coat were added.

† Degree of recovery was graded from 0 to 5 as follows: Degree 0: Tubules appear atrophic and contain only syncytium of Sertoli cells with few scattered spermatogonia. 1: Spermatogonia and spermatocytes present, but epithelium of seminiferous tubules contain only one or 2 layers of germinal cells. Polynucleated large round cells in advanced stages of dissolution may occur. Spermatids and sperm present. 2: Spermatogonia, spermatocytes and some spermatids present. Many spermatids are in stages of degeneration, and polynucleated cells may be formed by fusion of degenerating spermatids. Mature sperm are absent, whereas scattered maturing sperm may be seen. 3: Spermatogonia, spermatocytes, spermatids and a few sperm present. 4: Epithelium of seminiferous tubules appears normal, but number of sperm in lumen of tubules is less than normal. 5: Complete recovery.

‡ Calculated as elaidic acid.

unsaturation. $k_{233} = 0.65$ and $k_{268} = 0.04$. Methyl linolenate (*cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid methyl ester) was prepared by Dr. L. C. Norcia. Its iodine value was 251.5, $k_{233} = 1.6$ and $k_{268} = 0.09$. The infrared spectrum detected the presence of a small amount of *trans* unsaturation. Linseed oil had low ultraviolet absorption ($k_{233} = 0.4$ and $k_{268} = 0.08$) and its infrared spectrum showed no evidence of *trans* unsaturation.

Results. Growth. The fat-free diet allowed a weight gain during the period of supplementation only about half that caused by linseed oil or linolenate supplementation, and about two-thirds that caused by linoleate. Compared with rats fed fat-free diet, those fed linolelaidate or *cis, trans* linoleate grew very slowly. This growth inhibition was overcome in both cases by supplements of linseed oil. Elaidate allowed growth better than that allowed by the fat-free diet. Thus the single *trans* double bond in a monoenoic acid does not inhibit growth, whereas in the nonconjugated diene, linoleate, one or more *trans* double bonds induces inhibition. Previous studies of the action of these compounds with much older rats did not demonstrate actual inhibition of growth, but these acids were found unable to stimulate growth in the older deficient rats (3,4). In the present study, the effect is more striking.

Skin condition. The skin symptoms were worsened by supplementation with linolelaidate or *cis, trans* linoleate. Previous experiments with older rats had demonstrated the inability of these unnatural isomers to cure skin symptoms. The worsening of the skin symptoms was prevented by linseed oil when it was given with the unnatural isomers.

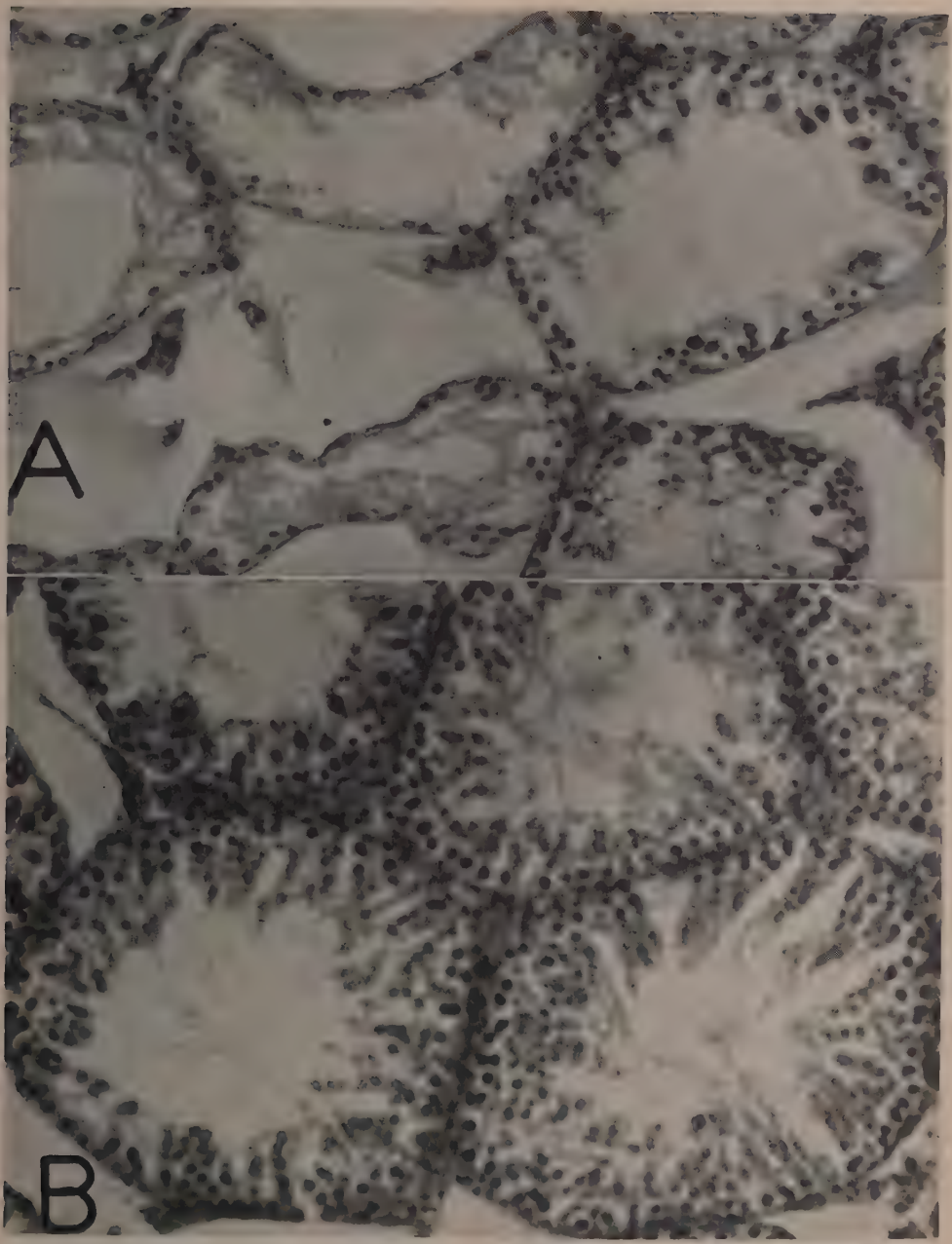


FIG. 1. A. Degeneration of spermatogenic tissue as result of EFA deficiency accelerated by cholesterol. Section of testis from rat No. 18. B. Recovery of spermatogenic tissue as consequence of supplementation with linseed oil. Section of testis from rat No. 16. Hematoxylin-Eosin stain. Magnification 67×.

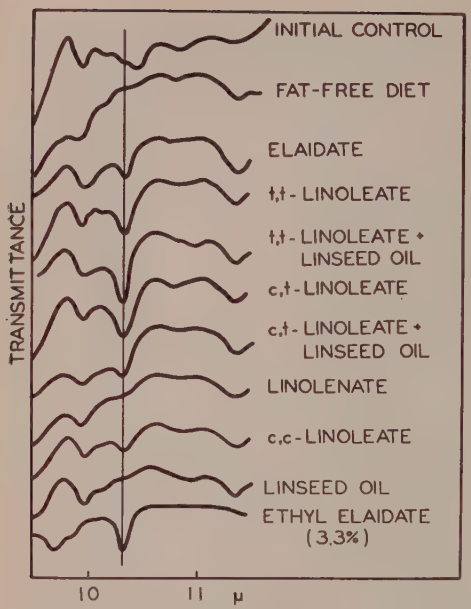


FIG. 2. Infrared spectra of fatty acid ethyl esters from rats fed various supplements. Spectra measured on 50% solutions of ester in carbon disulfide and compared with 3.3% ethyl elaidate + 46.7% ethyl oleate in carbon disulfide (lower curve).

Improvement of skin condition was significant only in the rats fed linoleate.

Testis weight. Testis weights of rats fed the *trans* isomers of linoleate were low. The ratio of testis weight to body weight was somewhat low in rats fed linolelaidate, in one rat fed *cis, trans* linoleate, in one fed *cis, trans* linoleate plus linseed oil and in one fed the fat-free diet. In all other cases only minor variations were found.

Histology of testis. That cholesterol increases the depletion of essential fatty acids is also indicated by the severe degeneration of spermatogenic tissue in the depleted rats. In a previous experiment weanling rats fed a fat-free diet 18 weeks had almost normal testicular tissue upon histological examination, but in the epididymi considerable numbers of degenerate spermatogenic cells were present (1). In the present study spermatozoa were not found in the epididymi of any of the rats. The epididymi were empty or contained only a few degenerate spermatogenic cells. Degenerated testes and the effect of feeding linseed oil are shown in Fig. 1. Supplementation of the fat-free diet with either elaidate or linolelaidate did not result in recovery of the spermatogenic tissue (Table I). Best recoveries were found in rats supplemented with linseed oil, and in one rat fed linoleate. Linolenate had no effect upon the spermatogenic tissue. The effect of *cis, trans* linoleate upon recovery of spermatogenic tissue is questionable.

Deposition of trans acids. The infrared spectra of methyl esters of the fatty acids of one rat from each group showed differences (Fig. 2). At the end of the depletion period there was *trans* unsaturation in the fatty acids of the rat. The sample taken from the fat-free rat at the end of the period of supplementation showed no evidence of *trans* double bonds, nor did the sample from the rat fed linseed oil. Fatty acids from the rats fed linoleate or linolenate had 1.2 and 0.9% of *trans* acids respectively, but the fatty acids from rats fed the unnatural *trans* isomers contained two or three times this amount. When the weight gain is related to the amount of *trans* unsaturation (expressed as elaidic acid), it is clear that the *trans* monoenoic acid does not have the growth inhibiting effect of *cis, trans* or *trans, trans* dienoic acids. It also appears that all the *trans* acids inhibited fatty acid synthesis.

Summary. Feeding weanling male rats a diet free of essential fatty acid (EFA), containing 1% cholesterol, 2% sulfasuxidine, and 1% hydrogenated coconut oil for 3.5 months induced an EFA deficiency accompanied by severe testicular degeneration. The degeneration was partially corrected by supplementation with linoleate or linseed oil for 3 weeks. However, sperm were not found in the epididymis in any case. *Trans* isomers of linoleic acid were unable to induce recovery of the testis. The *trans* isomers inhibited growth, worsened the skin condition, and were found to be deposited in the animal fat.

We are grateful to Dr. Jacques Chipault for aid in interpreting the infrared spectra and to Dr. James Peifer and Dr. J. P. Funch for valuable consultations.

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Histopathological Changes by Acetoacetate in Normal and Scorbutic Guinea Pigs.* (22700)

S. G. NAYUDU, SAILEN MOOKERJEA, AND M. C. NATH

University Department of Biochemistry, Nagpur, India

Acetoacetate, which on repeated injection has been reported from this Laboratory to induce experimental diabetes by causing inactivation of insulin(1), hyperglycemia accompanied with reduced glucose tolerance(2), depletion of blood GSH(3), depletion of thiamin, riboflavin and niacin(4,5), has recently been found to cause hypovitaminosis C(6), which is also known to bear a close relation with diabetes(7,8). It has also been shown that disturbance in carbohydrate metabolism brought about in scurvy(9) is further aggravated by continued injection of acetoacetate in gradually increasing doses(10). Now because acetoacetate causes depletion of blood ascorbic acid when injected in high doses and histopathology in scurvy has been shown by Mukherjee and Banerjee(11) to reveal widespread degenerative changes in liver, spleen, kidney, testes and suprarenal cortex of chronic and acute scorbutic guinea pigs and since β-hydroxy butyric acid, another member of the ketone bodies has been shown by Nath *et al.*(12) to bring about histological changes in the pancreas, it seemed desirable to study the histopathological changes of different tissues of normal as well as scorbutic guinea pigs injected with acetoacetate in gradually increasing doses.

Methods. Fifty male guinea pigs weighing from 300 g to 400 g were divided into 4 groups, each group containing animals of approximately corresponding weights. The last group of animals served as the control, each animal being fed 5 mg ascorbic acid daily supplementing the recommended scorbutic diet (Group I—Control). Group II animals received diet like that of Group I and plus daily injections of acetoacetate (Sodium salt solution of concentration 200 mg/cc at pH 7.2) intraperitoneally, in increasing amounts. The initial dose was 175 mg/kg, increased by 40 mg/kg every third day so that final dose after 21 days was 415 mg/kg (Group II—Acetoacetate injected). Scurvy was produced in animals of Group III by feeding scorbutic diet as recommended by Banerjee(13). Animals of Group IV were fed the scorbutic diet and injected daily with acetoacetate in dosage similar to that in Group II (Group IV—Scorbutic and superimposed with acetoacetate injection). Animals were housed in individual cages. Paired feeding technic was followed to obviate any results due to inanition. The animals of Group II served as the control for this purpose, as it was seen from a preliminary experiment that the diet intake of this group was found to be the lowest as the experiment advanced. The animals of this group were fed the scorbutic diet *ad libitum* and the amount consumed by each was noted every day. The same amount of diet was fed, to every animal of corresponding weight in all the other groups, on the subsequent day. Just after decapitation different tissues of the animals of each group were removed and fixed in Zenker-formol solution. After dehydration

* This investigation was supported by Research Grant from Council of Scientific & Industrial Research, Government of India.

and clearing, tissues were embedded in paraffin and 4-6 μ thick sections were cut and stained by haematoxylin and eosin stain. Pancreas were stained by Gomori's Phloxin-Haematoxylin method(14). Photomicrographs of different sections were taken under identical conditions.

Results. Histopathological changes in different groups of animals have been presented separately. Pancreas of the Group II animals (acetoacetate injected for 21 days) show an increase in the area of the islet tissues and the number of β-cells. Group III animals (Scurvy) show distinct early hypertrophy and early degranulative changes of the pancreatic islet cells but no degenerative changes. Group IV (Scurvy superimposed by repeated injection of acetoacetate) show more clear changes. Fibrosis and degranulation are more distinct. Hypertrophy of the islet cells and pyknotic changes in the nuclei, which precede degenerative symptoms of the β-cells are also evident. Animals of this group also show clear evidence of small degree of lipomatosis.

Kidney of Group II animals (acetoacetate injected) show hypertrophy of the glomeruli and early hyalinization of the proximal convoluted tubules. Changes in the tubular cells and nuclei are indicative of hyperfunction. Group III animals (scurvy) show atrophy of the glomeruli and degenerative changes in the tubules. Kidneys of the Group IV animals (scurvy superimposed by repeated injections of acetoacetate) show mild intercapillary glomerulosclerosis. Capsular cells are also distended. Proximal convoluted tubules show the presence of hyaline casts and necrosis.

Liver of the Group II animals (acetoacetate injected) show hydropic distension of the hepatic cells and pyknosis of a few nuclei around the central vein. In Group III animals (scurvy) hepatic cells show distinct signs of early degeneration. In some cases nuclei are found to be pushed to the periphery of the cells. Liver cells of the Group IV animals (scurvy superimposed by repeated injections of acetoacetate) show characteristic necrotic changes in the peripheral zone of the lobule. Hemorrhage of the hepatic cells is also seen around the central vein.

Discussion. Previous studies have shown that repeated injections of acetoacetate to guinea pigs kept on scorbutic diet cause a rapid development of hyperglycemia and depletion of glycogen storage of liver and leg muscle(10). Continued injection of acetoacetate alone can also cause different changes in carbohydrate metabolism similar to that observed in diabetic condition. Scurvy has been known to cause degranulation of the β-cells of the pancreas(15), and to lead to various disturbances in carbohydrate metabolism(9). Repeated injection of acetoacetate has also been found to destroy vit. C *in vivo* (6). The role of acetoacetate in the aggravation of the disturbances of carbohydrate metabolism in scorbutic condition(10) has thus been accounted for.

The present study of the histopathological changes in different tissues of the animals kept on scorbutic diet and simultaneously injected with repeated doses of acetoacetate is in keeping with the biochemical lesions reported earlier(10). Pancreas of the scorbutic animals show early degranulative changes. Acetoacetate injection alone can also cause hypertrophic changes but when both conditions are superimposed, histopathology of the pancreatic islet cells is considerably aggravated showing fatty infiltration and early fibrosis of the tissue and pyknotic changes of the nuclei indicating overactivity and exhaustion. Kidney changes also present similar results. Acetoacetate injection alone causes hypertrophy, showing hyperfunction of the tissue, indicating more work for the cortex to metabolize acetoacetate. Degeneration and atrophy of the tubules begin with scurvy; but scurvy superimposed with the injection of acetoacetate lead to intercapillary glomerulosclerosis a condition which appears commonly in clinical diabetes(16).

Hydropic degeneration of liver cells in acetoacetate treated animals may be due to toxicity of acetoacetate injections. Scorbutic hepatic cells show early degenerative changes, which are aggravated leading to necrosis and haemorrhage around the central vein when the acetoacetate injections are superimposed on scorbutic condition.

All these findings demonstrate the toxicity of acetoacetate injections upon the structural integrity of the tissues. They also confirm and explain the biochemical findings as regards the aggravation of disturbances in scurvy by acetoacetate(10). The accumulative disturbances in carbohydrate metabolism thus produced finally tend to affect the architecture of the pancreatic islets accompanied with the degeneration of the nuclei of the β-cells, commonly observed in clinical diabetes(17), thus confirming the deleterious effect of acetoacetate in inducing diabetes in experimental animals and disclosing a possibility of excess accumulation of acetoacetate serving as a prominent factor for gradual development of clinical diabetes.

Summary. The islets of Langerhans of scorbutic guinea pigs and those injected with acetoacetate were hypertrophied. Injection of acetoacetate into scorbutic animals caused further degranulation and degenerative changes. Acetoacetate brought about hypertrophy of renal cortex and scurvy caused atrophy of glomeruli. Combined treatment aggravated these degenerative changes. Both scurvy and acetoacetate caused central degeneration of liver and this is enhanced by the combined treatment. In agreement with histologic changes, biochemical findings indicate the toxicity of acetoacetate. Acetoacetate may be a prime factor in clinical development of diabetes.

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Purification of Pancreatic and Urinary Callicrein. (22701)

MARION E. WEBSTER, WILLIAM R. CLARK, AND PEARL ANDERSON
Department of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C.

The hypotensive substance callicrein (kallikrein or padutin) was first discovered by Abelous and Bardier(1) and systematically studied by Frey, Kraut Werle and co-workers for the next 3 decades(2-3). Procedures for the purification of both urinary and pancreatic callicrein(4-10), with the exception of that developed by Reisser(10), depend to some extent on the adsorption of impurities and/or callicrein on various colloidal salts. Since adsorption conditions are difficult to reproduce and frequently require the running of test samples before each step in the preparation,* the present method was devised based on an ethanolic fractionation scheme such as carried out by Cohn *et al.* upon plasma proteins(11) and utilized successfully by this laboratory for purification of hyaluronidases(12-13).

Methods. Callicrein content of the various fractions was determined by the method of

* Werle, E., personal communication.

Frey *et al.*(2). Dogs were anesthetized with 26 mg nembutal per kilo and the blood pressure recorded by a mercury manometer attached to the carotid artery. Injections of test solutions were made through a polyethylene catheter inserted in the femoral vein. Heparinized saline (50 mg per liter) was used to prevent clotting in the catheter. Samples of pancreatic callicrein containing ¼ to 2 Frey units† gave a response which was complete within one minute and solutions could readily be assayed at the rate of one every 2 minutes particularly with more highly purified preparations. However, with crude urinary fractions the blood pressure frequently did not return to its original level making the quantitation of the results difficult. *Total nitrogen* was determined by micro-kjeldahl digestion and direct nesslerization. For determinations where ammonium sulfate was present, aliquots were made alkaline with NaOH and the ammonia removed by aeration prior to digestion. Samples similarly treated, but not digested, served as controls. *Electrophoretic examination* and separation were conducted at 0-1° by moving boundary methods in the Perkin-Elmer equipment and the Aminco-Stern apparatus. Samples were equilibrated by dialysis with 0.1 ionic strength phosphate buffer pH 7.7.

Results. Purification of pancreatic callicrein: Fresh frozen bovine pancreas was thawed by standing overnight at 4°, then minced by machine. Three methods of extraction (autolysis(9), 0.1 M acetic acid and 0.25 M trichloroacetic acid) showed that 0.1 M acetic acid extracted the greatest number of callicrein units although autolysis gave the most highly purified product. Initial crude extracts were therefore prepared by addition of an equal weight of chilled 0.1 M acetic acid to cold mince. The preparation was stirred for 1 hour at 4° and then centrifuged at 2000 rpm and 26°. The extract was further clarified by filtration on Buchner funnels through filter paper pulp. The filtrate was measured and 1/10 its volume of concentrated buffer added to make 0.02 M phosphate at pH 5.5 containing 0.45% NaCl and 1:10,000 merthiolate. From 5 kg of pancreas a final volume of around 4,500 ml was obtained, containing 2.5-3 Frey units and 30-40 mg protein per ml as calculated from nitrogen assay.

First ethanol fractionation: The crude extract was adjusted to pH 5.5 and fractionated with ethanol at —5°. The ethanol added was precooled at —20° and introduced in a thin stream with concurrent stirring of the cooled buffered solution. After addition of each increment of alcohol, the fraction was allowed to equilibrate for at least 18 hours before removal of precipitate by centrifugation at 4000 rpm and —5°. The precipitate was dissolved in the minimum amount of buffer at 0°. Preliminary fractionation using 0.05 *mole fraction* increments of ethanol demonstrated that 50% of the callicrein activity could be recovered in the precipitate between 0.15 and 0.25 mole fraction with a 6-fold increase in purity based on nitrogen determinations. However, occasional losses of callicrein, due to adsorption on the large precipitate obtained with 0.15 mole fraction, led to eventual adoption of a one step precipitation at 0.25 mole fraction. As illustrated in Table I, this one step procedure effected a 3-4 fold increase in purification and retained all callicrein activity of original extract.

Second ethanolic fractionation: Since callicrein was known to be stable from pH 5.0 to 8.0(14) a pH of 7.5 was chosen for the next ethanolic fractionation. Experimentation revealed that callicrein was now precipitated between 0.25 and 0.45 mole fractions. Precipitate obtained from the first fractionation was dissolved in 0.02 M phosphate buffer and diluted to 10-12 mg protein per ml. The pH was adjusted to 7.5 and precipitates removed following addition of 0.25, 0.3 and 0.45 mole fractions. Excessive adsorption of callicrein on relatively large precipitates obtained with 0.25 mole fraction was not noted at this concentration of protein (Table I). Precipitates obtained from 0.3 and 0.45 mole fraction were

† The authors are deeply indebted to Dr. E. Werle, University of Munich, Germany, for supplying vials of standard callicrein containing 10 Frey units. These preparations contained 5.5-6 Frey units/mg and 45-55 Frey units/mg N.

TABLE I. Ethanolic Fractionation of Pancreatic Callicrein.*

Purification procedure	Conc. ethanol mole fraction	Total callicrein units	% recovered	Purity index, units per mg N
0.1 M acetic acid extract		12,800	100	0.5
1st ethanol fract. pH 5.5	.25	15,000	117	1.6
2nd ethanol fract. pH 7.5	.25	6,400	43	2.5
	.3	2,280	15	25
	.45	12,000	80	37
3rd ethanol fract. pH 6.5	.3	500	3.5	23.5
	.35	650	4.5	45
	.5	5,100	42.5	104
	.55	550	4.2	25

* Above data are based on avg figures obtained from five 5 kg batches of pancreas.

usually sufficiently small to warrant collecting in two centrifuge tubes before dissolving in buffer. This procedure was followed so that residual ethanol present in the cups would not destroy callicrein. The resulting solutions were about 20 times as pure as that obtained in first ethanol precipitation.

Third ethanolic fractionation: This fractionation was conducted on combined precipitates from 0.3 and 0.45 mole fractions from above. They were diluted in buffer to 0.5 to 0.75 mg protein per ml with the pH adjusted to 6.5. Even though the pH was nearer the isoelectric point (pH 4.0 to 4.3(2)), the decrease in concentration of protein being fractionated caused the bulk of activity to precipitate betwen 0.35 and 0.5 mole fractions (Table I). Following the three ethanolic fractionations approximately 5,000 Frey units could be obtained from 5 kg of bovine pancreas. The final preparation was approximately 200 times purer than initial crude extracts. Average yield of material calculated as protein was 400 mg. Callicrein of this purity was reasonably stable when stored at 4° and at 0.1% protein concentration. It is possible that variation in activity obtained from one preparation to another at this stage may in part reflect a certain instability. Crude acetic acid extracts could be dialyzed against running tap water without inactivation. However, material from the third ethanolic fractionation lost 50% of its activity in 24 hours dialysis. It could be dialyzed against buffer at 4° without loss.

Electrophoretic examination and separation. In order to obtain sufficient protein concentration for electrophoretic examination, 6,500 callicrein units which had been obtained by 3 ethanolic fractionations and which gave an average purity of 75 units per mg N were recombined in 350 ml, pH was adjusted to 5.5, and 0.3 mole fraction of ethanol added. The precipitate was extracted with 3 successive 12 ml portions of 0.1 ionic strength phosphate buffer pH 7.7. Approximately 75% of the enzyme was recovered in 3 solutions with 2-fold increase in purity for the first extract. The 3 solutions were separately dialyzed against buffer at 4°. No loss in total enzymatic activity was noted. The first extract showed another 2-fold increase in purity, the second extract went from 35 units per mg N to 150 units per mg N, while the third extract remained unchanged at 60 units per mg N. Each of the 3 solutions were analyzed electrophoretically by moving

TABLE II. Electrophoretic Separation of Partially Purified Callicrein.

	Total callicrein units	Purity index, units/mg N	Relative % of electrophoretic components					
			1	2	3	4	5	6
Extract No. 1	100	300	47	18	11	24	—	—
Ascending	75	430	69	19	6	6	—	—
Descending	7.5	85	18	18	18	45	—	—
Extract No. 2	850	150	27	22	6	13	8	23
Ascending	540, 540*	330, 297	42	29	6	11	4	8
Descending	61, 46	35, 37	14	16	6	15	11	38
Extract No. 3	100	60	14	9	2	5	5	65
Ascending	35, 75	320, 400	42	23	4	9	5	17
Descending	15, 30	38, 52	5	4	1	4	5	81

* Values represent duplicate determinations on same extract.



FIG. 1. Ascending electrophoretic pattern of partially purified callicrein (Extract No. 1). Time: 1920 sec. Volts: 5.0 volts/cm. Arrow indicates starting point.

boundary technic (Fig. 1) and then separated into 2 fractions, one containing the major portion of the fast component and the other the slow material with the intermediate substance being divided between the 2 fractions. As is shown in Table II, the sample with highest purity index, extract 1, showed 4 components with mobilities ranging from about —6 to —3 x 10^{-5} $cm^2sec^{-1}volt^{-1}$. The fastest component (as estimated from distribution pattern areas) accounted for 47% of the original material and the slowest 24%. Electrophoretic separation increased the concentration of fast material to 69%. The other two extracts contained 6 or more components varying in mobility from —6 to —0.5 x 10^{-5} $cm^2sec^{-1}volt^{-1}$. Their decrease in purity index paralleled the decrease in content of fast material and the corresponding increase in slower moving components. In addition, the activity of preparations following separation was invariably associated with the fast component. Callicrein, therefore, is tentatively identified as the most rapidly migrating substance present in these solutions. Activity of these purer preparations of callicrein, rapidly lost at room temperature, could be maintained only by storage of samples in frozen state. Further purification by moving-boundary procedure did not appear feasible since mobilities of various components were similar and diffusion rates were fast. Paper electrophoresis at 4° and 25°, could also be used for further purification of this enzyme. However, considerable loss was incurred both from irreversible adsorption on paper and by inactivation during time period required for the experiment.

Ammonium sulfate fractionation. Ammonium sulfate fractionations were carried out at 4° and individual precipitates were allowed to equilibrate for 24 hours before being removed by centrifugation at 4000 rpm, 4°. As is shown in Table III levels of purity were obtained which compare favorably with those achieved by electrophoretic separation. However, callicrein of this purity again readily lost activity at room temperature. The technic of adding albumin to maintain high protein concentrations which had been successfully used in stabilization of hyaluronidase up to 100,000 T.R.U. per mg N(15), failed

TABLE III. Ammonium Sulfate Fractionation of Partially Purified Pancreatic Callicrein.

Saturation of $(NH_4)_2SO_4$	Total callicrein units	% recovered	Purity index, units/mg N
.3	750	7.5	10
.4	1,200	12.0	50
.5	4,950	49.5	450
.6	1,500	15.0	300
Supernatant	750	7.5	5

to prevent inactivation of the hypotensive enzyme.

Purification of urinary callicrein. Methods originally devised for purification of bovine pancreatic callicrein were subsequently applied to purification of urinary callicrein from human urine. Urine was collected and stored for short periods of time under xylene. Addition of suitable amounts of 0.02 M phosphate buffer was made and pH adjusted to 5.5. Preliminary fractionation indicated that despite the increased dilution of enzyme in solution (0.075 Frey unit per ml as compared to 2.5-3.0 per ml for crude pancreatic extract) 90-100% of the enzyme was precipitated by the 0.25 mole fraction of ethanol. However to insure complete precipitation of callicrein 0.3 mole fraction was utilized. In 100 liters of human urine used in present studies, the average number of callicrein units was 0.75 Frey unit per 10 ml. This number of units is within the range found by previous investigators(2).

Callicrein in 40 liters of normal urine was concentrated by precipitation at pH 5.5 with 0.3 mole fraction. The solution was then diluted to 5 mg protein per ml as calculated from nitrogen assay, the pH adjusted to 7.5 and ethanol added to 0.15 mole fraction. Although the bulk of activity failed to precipitate until 0.3 mole fraction of ethanol, this stepwise separation was utilized in order to minimize loss by adsorption. The ethanol concentration was then raised to 0.25 mole fraction and a second inactive precipitate was removed. Precipitates from 0.3, 0.35 and 0.4 mole fractions were also obtained. As is shown in Table IV, the 0.35 fraction contained half the starting activity with better than a 50-fold increase in purity. Urinary callicrein differed from pancreatic in that precipitation at pH 7.5 occurred at a lower mole fraction despite an increased dilution of enzyme in solution (0.7 Frey unit per ml as compared to 3 units for pancreatic preparation).

Discussion. Although originally reported to be present in relatively large amounts in the saliva, pancreas and urine, more recent evidence by Werle and Maier(3) suggests that pancreatic and urinary preparations are chemically and pharmacologically different from the submaxillary substance. Callicrein is reported to exert its hypotensive effect by enzymatic action on callodinogen, present in normal plasma, to release a smaller molecule, presumably polypeptide, called callidin. In this manner the callicrein-callidin system with respect to hypotension can be compared to the renin-hypertensin (angiotonin) mechanism which has been associated with hypertension(16).

TABLE IV. Purification of Urinary Callicrein.

Purification procedure	Conc. ethanol mole fraction	Total callicrein units	% recovered	Purity index, units per mg N
Normal human urine		3,000		.01
1st ethanol fract. pH 5.5	.3	2,880	96	1.0
2nd ethanol fract. pH 7.5	.15	47.3	1.6	1.0
	.25	100	3.5	1.6
	.3	280	9.7	14
	.35	1,440	50.0	57
	.4	160	5.5	10

Callicrein has previously been obtained at high levels of purity by two authors(5,7) who utilized a variety of technics. The purity achieved by Bischoff and Elliot(5) from human urine is probably similar to that obtained by us with pancreatic callicrein. However, Kraut and Schweitzer(7) have reported preparation of callicrein solutions containing 2 micrograms of organic material per callicrein unit. Calculation of these data based on the assumption that a pure protein had been isolated would give a purity index of 3000 Frey units per mg N. On the other hand, if the fast component in electrophoretic analysis at pH 7.7 can be taken as a measure of callicrein, then a solution containing 700 Frey units per mg N should represent a homogenous preparation. The discrepancy between these calculations is so great that further work would be necessary to elucidate the difference. Those authors using bovine pancreas(9-10) attempted only preliminary purification of the callicrein. While it is undoubtedly true that more units of callicrein can be obtained by autolysis than by extraction with dilute acetic acid, in our hands, as well as those of Reisser(10), autolysis at 37°

gave secondary reactions which were undesirable and caused inconsistent results. The autolysis devised by Reisser which takes place for 72 hours at 2° has not been attempted in this laboratory since it was desired to take all possible precautions against contamination of the extracts. However, this method of extraction should cause a 4-fold increase in the recoverable units.

The isolation of callicrein as a chemical entity would appear to depend on the finding of a method for stabilizing this hypotensive substance. The instability encountered in our preparation at around 400 Frey units per mg N and those reported by other authors(5,7) has hampered further attempts to characterize this enzyme.

Summary. A method has been devised for preparation of highly purified pancreatic callicrein. The fractionation procedure involves three ethanolic fractionations at pH 5.5, 7.5, and 6.5 followed by one ammonium sulfate precipitation. Solutions of hypotensive enzyme at this level of purity proved highly unstable and further purification was abandoned. Preliminary purification of urinary callicrein revealed that the method could be adapted to this source.

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Tolerance of the Rat for N,N′-Diphenyl-P-Phenylenediamine. (22702)

H. H. Draper, S. Goodyear, K. D. Barbee, and B. C. Johnson

Division of Animal Nutrition, University of Illinois, Urbana

The demonstration that N, N′-diphenyl-p-phenylenediamine (dppd) is effective in the prevention of encephalomalacia in chicks fed vit. E-low diets containing fish oils, and in the stabilization of carotene and vit. A in practical rations, has led to its widespread use in commercial mixed feeds. The concentration commonly employed is 0.025% of the diet. Matterson *et al.*(6) have reported that chicks which received a diet containing 1% dppd throughout a 12-week growth trial remained free of serious lesions and maintained a normal growth rate. Draper and Johnson (1) observed no adverse effects from administering dppd to lambs at a level of 0.1% for 7 weeks, and Jensen and coworkers* reported that 0.025% dppd improved reproductive performance in turkey hens fed a tocopherol-low ration.

The present experiments were designed originally to determine the degree of replace-

* Abstract published.

TABLE I. Reproductive Performance of Rats Receiving a Vit. E-Free Diet Supplemented with dppd from Weaning Age.

Treatment	No. females	No. conceptions	No. pups born		% born alive
			Total	Per conception	
0	25	0	0	0	—
Vit. E (30 mg/wk)	19	17	155	9.1	57
.1% dppd	10	7	21	3.0	19
.025% "	14	10	71	7.1	7
.005% "	16	16	108	6.8	14
.385 mg " /wk*	10	9	69	7.7	88

* Approximately .0006% of diet.

ability of vit. E by dppd in the rat; but following the observation that small concentrations of dppd fed continuously through growth and gestation produced adverse effects upon reproduction, a study of the long-term consequences of dppd feeding was conducted.

Methods. Weanling female rats of the Sprague-Dawley strain were maintained throughout growth and reproduction on a diet of the following composition (%): Cerelose 64.6, casein 20, tocopherol-low lard 10, salts(5) 4, choline 0.4, vitamin premix (in cerelose) 1. Vit. A and D were administered weekly by dropper. The premix provided the following concentrations of vitamins in μg per gram of diet: Thiamin 100, riboflavin 15, nicotinic acid 100, pyridoxine 6, calcium pantothenate 40, biotin 0.5, menadione 4, folic acid 4 and vit. B_{12} 0.05. Comparable groups of rats were fed the basal diet alone or supplemented with alpha-tocopheryl acetate (approximately 30 mg/wk placed on the diet), or dppd (0.1%, 0.025%, or 0.005%). Other animals received .385 mg dppd per week in 3 equal oral doses (equivalent to approximately 0.0006% of the diet eaten).

Results. During the growing period of 6 weeks all animals gained normally and remained in healthy condition with no manifestations of dppd intoxication or vit. E deficiency. The females, weighing 175-200 g, subsequently were mated to healthy males which had been maintained on a stock diet.

During gestation the females receiving 0.1% dppd exhibited anorexia, rough haircoat and anemia. At parturition gross hemorrhages from the vagina were observed and, upon autopsy, hemorrhages were located in the uterus. Of 10 females, 3 succumbed to hemorrhages and the remainder were in semimoribund condition. The hemorrhagic animals were acutely anemic but the prothrombin times were normal. Seventeen dead and 4 live pups were obtained from this group.

Improvement in reproductive performance was observed with lower concentrations of dppd in the diet. These results are summarized in Table I. Litter size was comparable among the groups receiving the 3 lowest levels, but the proportion of live pups was subnormal except at the lowest concentration. No abnormalities were found in the hemoglobin values, prothrombin times or white blood cell counts during gestation. However, the feeding of dppd appeared to be associated with an increase in the gestation period, the average for the females receiving 0.025% dppd being 25.4 days and that for the 0.005% level being 23.8 days, compared with 22.8 days for the positive controls.

The efficacy of dppd in reversing sterility in females maintained on a vit. E-low diet was also examined. The females were depleted of vit. E using a diet similar to that outlined but containing 4% fish liver oil as a source of fat. Methyl linoleate was given orally twice per week at a rate equivalent to 0.4% of the diet. All the females exhibited reproductive failure during the first cycle. Supplementation was begun one week before remating. The results are shown in Table II.

It has been reported previously that dppd fails to regenerate vit. E-deficient female rats fed a diet essentially devoid of this vitamin, although, at a level of .385 mg per week given prophylactically, dppd can delay the appearance of resorption-gestation for one or two cycles(2,3). The regenerative effect observed

TABLE II. Efficacy of dppd in Restoring Fertility of Female Rats Previously Maintained on Vit. E-Low Diet.

Treatment	No. females	No. conceptions	No. pups born— Total	No. pups born— Per conception	% born alive
0	22	0	0	0	—
.1% dppd	19	15	71	4.7	3
.01% "	17	17	109	6.4	5
Vit. E	23	19	161	8.5	71

in terms of pups born in this experiment is probably attributable to its protective action on the small quantity of vit. E contributed by the fish liver oil. The oil, upon assay by the procedure of Tosic and Moore(4) was found to contain 8 mg/100 g, and contributed, therefore, about 3.2 μg/g of diet. These data indicate that the dietary requirement of the rat for vit. E may be considerably reduced when an effective antioxidant is supplied simultaneously, and support the concept of a dual role for vit. E in the body, including a general antioxidant function and another indispensable role in whch it cannot be replaced by other antioxidants. The inferior survival *in utero* on both concentrations of dppd in this regeneration experiment further substantiates the long-term toxic effect of this compound at levels well below those used in commercial feeds. However, only slight indications of hemorrhaging at parturition were observed in the animals receiving 0.1% dppd, possibly because of the shorter period of administration. The possibility that levels below .01% might exert a regenerative effect is being investigated.

Weanling rats showed no adverse effects from receiving 0.5% dppd in the diet for 12 weeks, but subsequently developed "porphyrin" staining of the whiskers and haircoat over the head and neck. This condition was accompanied by a rough haircoat and moderate depression of weight gain. Determinations of hemoglobin values and prothrombin times on blood from these animals have revealed no significant changes. Food consumption and growth rate during the early part of the growing period indicate that palatability was not a problem.

In the majority of these experiments the dppd employed was a feed grade product containing approximately 95% active material. However, part of the growth and reproduction studies were conducted using pure dppd, and indicate that the toxicity also is encountered after prolonged feeding of the pure compound and is not attributable to a contaminant in the feed grade product.

The results of this study show that prolonged continuous oral administration of dppd in a vit. E-low diet at levels substantially below those used commercially in mixed feeds is toxic for the rat. The symptoms do not appear during growth, except at very high concentrations, but occur at parturition, and are characterized mainly by vaginal hemorrhages in the female and a high incidence of stillbirths. The possibility that this toxicity may be significantly modified by the presence of vit. E in the diet is worthy of investigation but may be regarded as unlikely.

Summary. Inclusion of N, N′-diphenyl-p-phenylenediamine in a vit. E-low diet fed to female rats through growth and reproduction has resulted in varying degrees of reproductive failure. Toxicity was encountered during growth at a level of 0.5% but not at 0.1% or below. Rats receiving 0.1% dppd exhibited uterine and vaginal hemorrhages at parturition accompanied by anorexia, anemia and emaciation. Concentrations of 0.025%, 0.01% and 0.005% resulted in a high incidence of stillbirths. No adverse effects were encountered from feeding 0.0006% dppd in the diet. The data show that dppd elicits symptoms of toxicity in the rat at concentrations below those used in commercial mixed feeds.

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Metabolic Properties of Quinidine; Effects of Quinidine Sulfate on Anaerobic Carbohydrate Metabolism of Rat Diaphragm.* (22703)

H. A. ELLS, R. CAPUTTO, AND R. H. FURMAN

Cardiovascular Section, Oklahoma Medical Research Foundation, the Departments of Medicine and Biochemistry, University of Oklahoma School of Medicine, Oklahoma City, and Department of Zoology, University of Oklahoma, Norman

Several papers have appeared recently concerning the effect of quinidine on various aspects of carbohydrate metabolism(1,2,3). The influence of this compound on the anaerobic phase of carbohydrate metabolism has not been thoroughly explored, and results which appear to be conflicting have been reported. Following the work of Furman and Howard(4) confirmed by Fajans and his group(5) who showed that quinidine decreases glucose tolerance in human subjects, we decided to investigate the influence of this drug on the anaerobic metabolism of rat diaphragm in an attempt to gather information on the mechanism of this effect.

The combined results of these experiments on the effect of quinidine on endogenous glycolysis, on glycolysis in the presence of glucose and fructose and on anaerobic consumption of glucose and fructose, point to the conclusion that utilization of these hexoses is impaired at one or more steps close to their entry into the Embden-Meyerhof sequence.

Materials and methods. Albino rats (Holtzman) weighing from 90 to 130 g were used. Following a 24-hour fast the rats were killed by a blow on the head and the diaphragms quickly removed and placed in Krebs-Ringer's bicarbonate solution at 1°C. The diaphragms were cut into either 2 or 4 pieces, weighed and used as soon as possible. All experiments were carried out under anaerobic conditions using the standard Warburg procedure. Incubation was carried out at 37°C in Krebs-Ringer's bicarbonate solution with or without quinidine sulfate (U.S.P., Merck). The flasks were gassed one-half hour with 95% nitrogen-5% carbon dioxide. After an equilibration period of 15 minutes glucose (Baker) or fructose (Nutritional Biochemicals) dissolved in Krebs-Ringer's bicarbonate solution was added from the side bulb to give a final total volume of 3.0 ml. Glucose or fructose was determined by the Somogyi-Nelson method (6).

Results. Effect of quinidine on anaerobic uptake of added glucose and fructose. Glucose (4 mg/ml) and fructose (5 mg/ml) uptake was determined, each in a series of 5 experiments, with or without 1×10^{-3} M quinidine. The following mean values and standard errors were obtained: glucose consumption without quinidine, 2.74 ± 0.21 mg/g (wet wt) tissue/hr; with quinidine, 1.44 ± 0.16 mg/g/hr; fructose consumption without quinidine, 2.70 ± 0.27 mg/g/hr; with quinidine, 1.28 ± 0.08 mg/g/hr. The inhibition of glucose or fructose uptake produced by 1×10^{-3} M quinidine is significant at the 0.01 level. In 2 experiments in which glucose consumption was measured for a 30-minute pe-

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TABLE I. Effect of Quinidine Sulfate on Anaerobic Glycolysis of Rat Diaphragm. Figures are mean values and ranges for CO_2 production in μl/g tissue (wet wt)/hr in Krebs-Ringer's bicarbonate solution. Incubation period 2 hr.

Substrate, mg/ml	No. of exp.	No quinidine		Quinidine concentration	Quinidine		Change in endogenous With quinidine	Stimulation by substrate No quinidine	Stimulation by substrate Quinidine
		Endogenous	With substrate		Endogenous	Substrate		(%)	
Fructose 5	8	100 (77–133)	109 (84–135)	1×10^{-3} M	94 (70–123)	93* (67–106)	- 6	+ 9	- 1
Glucose 1	2	112 (106–118)	162 (155–169)	5×10^{-4} M	105 (99–110)	130 (125–135)	- 6	+ 45	+24
" 1	3	90 (80–99)	132 (108–178)	1×10^{-3} M	88 (83–94)	83 (71–92)	- 3	+ 47	- 6
" 4	2	70 (63–77)	144 (122–167)	1×10^{-3} M	73 (68–77)	78 (75–81)	+4	+106	+ 7

* 5 experiments.

riod the inhibition was less marked: 7.10 mg/g/hr without quinidine; 4.80 mg/g/hr with quinidine.

Effect of quinidine on lactic acid production. The effect of quinidine on the endogenous anaerobic $Q_{CO_2}^{N_2}$ of rat diaphragm and on $Q_{CO_2}^{N_2}$ in the presence of added glucose and fructose is shown in Table I. Endogenous lactic acid production is not affected by 1 x 10^{-3} M quinidine. The results indicate a marked stimulation of lactic acid production by added glucose at concentrations of 1 mg/ml or 4 mg/ml. This stimulation is abolished by 1 x 10^{-3} M quinidine. Quinidine at a concentration of 5 x 10^{-4} only partially inhibits the stimulation of glycolysis by 1 mg/ml of glucose. Added fructose at a concentration of 5 mg/ml produces, unexpectedly, only a slight increase in lactic acid production over the endogenous level. In the presence of added fructose and 1 x 10^{-3} M quinidine, lactic acid production is again reduced to the endogenous level.

Discussion. The experiments reported here show that 1 x 10^{-3} M quinidine inhibits the uptake of added glucose and fructose by rat diaphragm under anaerobic conditions and abolishes the stimulation of glycolysis by glucose and fructose. Endogenous lactic acid production in skeletal muscle has been shown to be at the expense of stored glycogen(7,8). Our observation that quinidine has no effect on endogenous anaerobic glycolysis in intact rat diaphragm suggests that quinidine does not affect any of the metabolic steps between glycogen and lactic acid and therefore inhibits glucose or fructose uptake prior to the step at which these sugars and glycogen share a common metabolic pathway. This suggestion finds support in the work of Uyeki, Geiling and Dubois(3) who found that quinidine did not inhibit lactic acid production from fructose-1, 6-diphosphate in rat heart homogenates.

The inhibitory effect of quinidine on glucose uptake found in the present experiments may account for the decrease in human glucose tolerance noted following quinidine administration(4,5).

Summary. 1. Quinidine sulfate at a concentration of 1 x 10^{-3} M strongly inhibits the uptake of glucose and fructose by intact rat diaphragm under anaerobic conditions. 2. Quinidine is without effect on endogenous anaerobic lactic acid production by rat diaphragm. 3. Glucose at a concentration of 1 mg/ml or 4 mg/ml markedly stimulates anaerobic glycolysis while fructose at a concentration of 5 mg/ml produces only a slight stimulation. 4. Stimulation of glycolysis by glucose or fructose is abolished by 1 x 10^{-3} M quinidine and partially inhibited by 5 x 10^{-4} M quinidine. 5. It is suggested that quinidine inhibits glucose or fructose uptake prior to the step at which these sugars and glycogen share a common metabolic pathway.

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In vitro Effect of Two Pantheine Analogues on Co-Enzyme A Requiring Systems.* (22704)

L. S. DIETRICH AND D. M. SHAPIRO (Introduced by David V. Habif)

Departments of Biochemistry and Surgery, College of Physicians and Surgeons, Columbia University, New York City

The use of metabolic analogues in the study of intermediary metabolism is a method of long standing. That an antagonist of a metabolite active in a multitude of systems would affect each system to varying degrees is predictable in view of the variation in physical constants pertaining to substrate-enzyme, apo-enzyme—co-factor relationships. In the field of vitamin metabolism, experimental data supporting this prediction has only recently been forthcoming(1-4). Analogues have been found which have a very striking antagonizing effect on one or more pathways dependent on the normal metabolite. The same concentration of drug, however, has little effect on other such dependent systems (3,4). These extremes in affinity of metabolic analogues for various systems in which the normal metabolite participates are of great importance in regard to chemotherapy. The present communication extends these observations to analogues affecting pantothenic acid metabolism.

Experimental studies pertaining to the *in vitro* effect of 2 pantethine analogues, omega-methylpantethine and bis (beta-pantoyl-aminoethyl) disulfide on the ability of pigeon liver homogenates to acetylate sulfanilamide and to synthesize citrate from fumarate and pyruvate are presented in the Table. Although omega-methylpantethine inhibited sulfanilamide acetylation, a similar concentration of this compound was without activity against citrate formation. Increasing the concentration of omega-methylpantethine did, however, have some inhibiting effect on the ability of pigeon liver to synthesize citrate from pyruvate and fumarate. The addition of bis (beta-pantoylaminoethyl) disulfide to an aliquot of the same homogenate inhibited citrate formation. At this same concentration little effect was observed on sulfanilamide acetylation. Increasing the level of bis (beta-

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TABLE I. Effect of Omega-Methylpantethine and Bis (beta-Pantoylaminoethyl) Disulfide on Sulfanilamide Acetylation and Citrate Formation in Tissue Homogenates.*

Treatment		% original activity† Sulfanilamide disappearance‡	Citrate formation‡
None		100	100
Omega-methylpantethine	1.8×10^{-3}M	52	96
	5.5 "	46	86
Bis-(β-pantoyl) disulfide	1.8 "	91	51
	5.5 "	68	15

* All values are avg of 2 separate experiments run in duplicate.

† Control values were 2.68 μM sulfanilamide acetylated/hr/g wet wt in the case of sulfanilamide studies and 28.4 μM citrate formed 50 min./g tissue wet wt in the case of the citrate studies.

‡ Each reaction vessel contained: 250 mg pigeon liver, 0.01 M Mg^{++}, 0.154 M K^+, 0.02 M phosphate, 0.003 M ATP, 4.8×10^{-3}M L-cysteine, 0.03 M acetate, 5.8×10^{-6}M sulfanilamide and pantothenic acid analog as indicated, in a final vol of 2.9 ml. All components were adjusted to pH 7.4.

Each reaction vessel contained 40 mg pigeon liver, 0.01 M Mg^{++}, 0.154 M K^+, 0.02 M phosphate, 0.003 M ATP, 2.4×10^{-3}M L-cysteine, 0.03 M pyruvate, 0.03 M fumarate, 3.5×10^{-3}M sodium monofluoroacetate and pantothenic acid analog as indicated in a final vol of 2.9 ml. All components were adjusted to pH 7.4.

pantoylaminoethyl) disulfide produced inhibition in the acetylation process. Thus, with 2 analogues at the proper concentration, a striking inhibitory effect on one Co-enzyme A dependent system is observed with only slight interference with another Co-enzyme A dependent system.

Co-enzyme A competitively protects transacetylase from inhibition by high concentration of bis (beta-pantoylaminoethyl) disulfide (1). We have confirmed these findings and have extended them by showing that Co-enzyme A protects both this system and that concerned with citrate formation against antagonism by either omega-methylpantethine or bis (beta-pantoylaminoethyl) disulfide (unpublished data). Thus, the mechanism of *in vitro* inhibition observed in the above cases, may be attributed to competition between the normal mercaptan, Co-enzymes A, and the related compounds, omega-methylpantethine and bis (beta-pantoylaminoethyl) disulfide.

It must be pointed out that the *in vitro* studies reported probably bear little relationship to the mechanism of carcinostasis observed with both of these compounds(5), since tumor inhibition is obtained with drug concentrations far below the levels used *in vitro*.

Summary. Omega-methylpantethine was observed markedly to inhibit sulfanilamide acetylation at concentrations which were ineffective against citrate formation in pigeon liver homogenates. With aliquots of the same homogenate, bis-(beta-pantoylaminoethyl) disulfide was observed to inhibit citrate formation at concentrations which were ineffective against sulfanilamide acetylation. In either case, higher concentrations of these compounds were effective in inhibiting both systems.

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Determinants of the So-called "Prothrombin Time".* (22705)

JOHN H. FERGUSON AND MARY JANE PATCH
Department of Physiology, University of North Carolina, Chapel Hill, N. C.

A recent study(1), assaying the blood-clotting components in normal newborn infants, emphasizes the lack of bleeding disorders and the essentially normal values of the unmodified "prothrombin time" test(2), despite significantly low levels of prothrombin and proconvertin (SPCA, stable factor, factor VII, etc.). It was noted, however, that proaccelerin (AcG, labile factor, factor V, etc.) in the infants' bloods, contrary to previous reports(3,4) was usually *hyper*normal, with values up to 300% of standard normal adults' levels. Dr. Quick(5), and others, have recognized the influence upon the one-stage prothrombin time test of *hypo*normal levels of all 3 of the above thrombin-forming factors. Recently, Quick and Hussey(6) attempted to test effects of adding "excess" of (a) AcG (labile factor) or (b) proconvertin (stable factor) to (1) normal and (2) prothr.-deficient, (3) AcG-deficient, and (4) proconv.-deficient plasmas. They concluded that " . . . a deficiency of any one is *not* compensated by an excess of the other two . . ." (*i.e.* of the 3 thrombin-forming factors). " . . . The determinant of the prothrombin time is the component which decreases below a minimum critical level . . ." As these authors do not give analyses to show the exact amounts of the 3 components in each test, there is considerable question as to the degrees of the deficiencies and "excesses" stated. The following experiments, therefore, were designed to retest various artificial plasma systems, whose assayed composition with reference to the 3 factors under consideration was defined, and could be correlated with the clotting-times after adding calcium and tissue thromboplastin essentially in the manner of the Quick test.

Methods and reagents. Assays: I. (pro) thrombin, (pro) convertin, and (pro) accelerin—the method does not distinguish precursors from the activated forms—are assayed by specific onestage procedures, which follow a common pattern, thus: 0.1 ml of suitable dilution of agent to be tested is mixed with 0.1 ml appropriate "substrate" and the clotting timed at 37°C on adding 0.2 ml *Catpln.* (equal (0.1 ml) vols., preincubated, of 0.02 M $CaCl_2$ and 'Soluplastin', courtesy of Schieffelin & Co.). The *substrates* are modified plasmas lacking the factor to be tested, but complete with regard to the other two(1). Clotting-times are converted into *percentage* units by reference to standard dilution curves obtained with normal adult human plasmas. These "units" (per ml) are employed throughout the present work. Confirmatory prothrombin analyses by the 'improved' 2-stage method(7) are also performed, but give no significant differences in the assay values.

II. *Thrombin* test: 0.2 ml fibrinogen + 0.2 ml agent tested. Observe for clotting.

III. *Antithrombin test*: Incubate 1 ml agent + 1 ml thrombin (bovine, Upjohn's, 20 units/ml) in siliconed tubes, at 25°C, and test 0.2 ml samples with 0.2 ml fibrinogen, at intervals, to observe any loss of thrombic activity.

Materials. Oxalated dog plasma is adsorbed with $BaSO_4$, 100 mg/ml, for 10 min. at 25°C. The supernatant is used to prepare *fibrinogen* by ¼ sat. $(NH_4)_2SO_4$, redissolving the precipitate in ⅓ original vol. of 0.85% NaCl and dialysing against saline containing 0.05 M trisod. citrate. This stock fibrinogen solution is stored frozen at —20°C for many months and is free from prothr., proconv., proaccel., by the above assays.

The $BaSO_4$ sediment is washed (x 2) with dist. water and eluted with ⅓ original (plasma) vol. of 0.2 M trisod. citrate and the *eluate* dialysed against 0.85% NaCl. *Eluate assays. Prep. I.* Prothr. 180, proconv. 100; AcG trace (±), thrombin 0, antithrombin 0. *Prep. II.* Prothr. 120; proconv. 174; AcG

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TABLE I. Varying AcG, at Several Eluate (Prothrombin + Proconvertin) Dilutions.

Eluate (I)	Pro-thrombin	Procon-vertin	AcG ±	15	30	60	100	200	300
			c.t. (sec.)						
1 : 1	60	33.3	38.2	20.2	18.1	13.7	11.5	*10.6*	*10.2*
1 : 2	30	16.7	48.1	28.1	22.6	16.4	13.9	11.8	*10.4*
1 : 4	15	8.3	64.4	—	23.1	21.6	15.7	14.9	13.7
1 : 8	7.5	4.1	92.	—	—	28.5	23.6	18.5	17.9

±; thr. 0; antithr. 0. *AcG* ('serum'-type or accelerin): $BaCO_3$ adsorbed (4 g/100 ml) beef serum (8), assaying 1240-1380 AcG, and free from proconvertin and all but a very insignificant trace of (pro) thrombin. It contains antithrombin. *Proconvertin* (Prep. A). Serum obtained from recalcified oxalated beef plasma after 18 hrs at 4°C is treated with $BaSO_4$ (10 g/100 ml), the sediment washed (x 4) with dist. water and eluted with 1/5 original vol. of 0.2 M trisod. citrate and the eluate dialysed. This preparation assays 85 *pro*convertin, which special tests show to be mostly in the precursor form, as in the 'prothrombin'-eluates. Other assays: prothr. ±; AcG 0; thr. 0; antithr. ±. Many tests were made with this preparation, but the results were so similar to those with prep. B, in the cited experiments, that they will not be detailed. *Convertin* (Prep. B). Dr. R. H. Wagner *et al.*(9) of our Pathology Dept., kindly supplied us with this preparation, termed (SA) "serum accelerator" in their cited publication in this journal. Our assays show: convertin 81; prothrombin 1; AcG 0; thr. 0; antithr 0.

Test method. Using the above assay values and suitably diluting and mixing the reagents, various *artificial plasmas* are reconstituted. 0.15 ml (*e.g.* 0.05 ml eluate + 0.05 AcG or (pro) conv + 0.05 ml fibrinogen) is tested with 2 vols (*i.e.* 0.3 ml) Ca-tpln., at 37°C, in essentially the manner of the "prothrombin time" test. Such test on 0.15 ml normal dog plasma gives clotting-times of 7-8 seconds.

Results. Table I shows effects of varying the AcG, with 4 different dilutions of eluate (prothr. + proconv.). In each case clotting-times (c.t.) get shorter with increasing AcG. There is a significant failure to find any true "optimum" of AcG concentration. Thus c.t.'s continue to shorten above the 'normal' (100) AcG level even to 3 times that strength. When there is enough prothrombin (and proconvertin), the shortest c.t.'s tests, namely at 300 AcG in the 1:1 and 1:2 dilutions of eluate, are so similar as to be within the experimental error of reading. This is so despite the fact that one has twice the prothrombin (and proconvertin) content of the other. Under these circumstances, the test obviously ceases to be a measure of prothrombin (and proconvertin).

Table II shows effects of varying (pro) convertin (added prep. B), again at 4 different dilutions of eluate. Parallel tests are performed with added excess (300) AcG and with merely the trace (±) of AcG present in the eluates. Clotting-times are shorter, in each series, the higher the (pro) convertin level and the less dilute the prothrombin. The chief conclusion from this experiment is that increasing the (pro)-convertin, up to 85, fails to compensate for lack of AcG or diminishing amounts of prothrombin.

Table III is similar to the foregoing in varying the (pro) convertin and AcG, but the prothrombin is kept at a constant very low level. In each series, there is here a definite small improvement with each incre-

TABLE II. Varying Proconvertin, at Several Eluate Dilutions, with Excess or Trace Amounts of AcG.

Eluate (II)	Pro-thrombin	(Pro)con-vertin	AcG	Clot.-time
1 : 1	40	85	300	8.7″
"	"	"	±	17. "
1 : 2	20	56	300	9.3″
"	"	"	±	17.8″
1 : 4	10	41.5	300	11.5″
"	"	"	±	22.6″
1 : 8	5	34.3	300	13.4″
"	"	"	±	28.3″

TABLE III. Varying (Pro)convertin, at Constant Very Low Level Prothrombin (5), and with Excess or Trace Amounts of AcG.

	(Pro)convertin	AcG	Clot.-time
1 a	34.3	300	13.4″
b	34.3	±	28.3″
2 a	20.8	300	14.4″
b	20.8	±	30.6″
3 a	14	300	15.3″
b	14	±	34.2″
4 a	7.3	300	18.2″
b	7.3	±	40. ″

ment of (pro)convertin, in a range of rather low levels. However the large differences due to the major AcG variable persist.

Table IV shows effects of varying (pro) convertin (added prep. B), at constant low prothrombin level, with 4 different levels of AcG. In each case there is a definite inhibitory effect at the highest (69.5) (pro)convertin concentration and least effect at the lowest (29, proconvertin in eluate only), with an apparent optimum (at 56) in the first 3 series. At the highest (200) AcG levels, the effects of varying the (pro)convertin are abolished except for the minor inhibition. The differences between the 4 series which depend upon the AcG level are not significantly altered by varying the proconvertin. In fact, the effects due to the variation of the proconvertin level are quite minor in the range studied.

Table V shows effects of varying (pro)convertin (added prep. B), with constant 'normal' (100) AcG, at 2 fixed concentrations of prothrombin. In 1a the small improvement over 1b (at ½ the (pro)conv. conc.) is real. In 2b the improvement due to increase of (pro)conv. over that in 2c is again significant. In 2a the inhibitory effect of too much added convertin begins to appear (*cf.* Table IV).

TABLE IV. Varying (Pro)convertin, with Low Constant Prothrombin, at Different AcG Levels.

	AcG	Pro-thrombin	(Pro)convertin* 29	42.5	56	69.5
			c.t.			
1.	±	20	17.0	15.6	15.1	18.5
2.	13.3	20	13.5	11.6	11.2	14.0
3.	66.7	20	10.9	10.0	9.8	10.7
4.	200	20	*9.7*	*9.7*	*9.8*	10.3

* 29 units are *pro*convertin in the eluate, the rest is *convertin* added.

Discussion. Besides confirming the now well established fact that these one-stage clotting times (*cf.* "prothrombin time") depend upon the 3 known variables, prothrombin, AcG, and proconvertin, the present experiments add new information on the relative importance of each and on the results of varying their quantitative interrelationships upon the observed clotting-times.

Most significant is the evidence (Tables I and IV) that in a certain moderately low range of prothrombin (and proconvertin) concentrations a truly large excess, *i.e.* 200-300% of 'normal', AcG can abolish the clotting-time differences, within an experimental error of a few tenths of a second, which would be seen at 100% or lower AcG concentrations and, if shown, be an index of the varying prothrombin (and proconvertin). The AcG level is a very significant variable. On the other hand, the (pro)convertin, at any fixed prothrombin or AcG level, exerts relatively minor effects although these are not insignificant. Increasing amounts of (pro)convertin do compensate somewhat for reduced prothrombin levels, but very incompletely and they do not at all abolish the large differences due to varying AcG. There is an experimental limit to our tests on these points due to the appearance of an inhibitory phenomenon. This could not be attributed to antithrombin, since it was not seen with excessive amounts of the AcG preparation which was rich in that factor. It was seen even more in the non-cited experiments with our own *pro*convertin (prep. A), which did have a small amount of antithrombin. The antithrombin test with Dr. Wagner's convertin, however, was negative,

TABLE V. Varying (Pro)convertin, with Constant Normal (100) AcG, and 2 Fixed Concentrations of Prothrombin.

	AcG	Pro-thrombin	(Pro)convertin*	c.t.
1 a	100	40	94.8	9.3″
b	100	40	58	9.9″
2 a	100	20	80.3	10.9″
b	100	20	51.3	10.1″
c	100	20	29	12.0″

* In 1 series, 58 units are *pro*convertin in eluate; in 2 series, 29 units are *pro*convertin in eluate.

but it, too, gave inhibition (see Tables). Whether these inhibitory effects and the apparent "optimum," for concentrations of (pro)convertin, are due to (a) some unidentified inhibitor contaminating the present preparations, or (b) some type of competitive reaction in the complex sequence of the thrombin-forming processes, are matters which will require further investigation.

Further technical questions may arise concerning possible difference in one-stage systems, between *pro*-accelerin or *pro*convertin and their 'activated' forms, accelerin or convertin, respectively. Nothing in our test system suggests such a possibility and the determinants we have sought by adding the 'activated' forms, in all probability, hold also for their precursors. We believe, therefore, it is reasonable to transfer the results of these experiments to interpretations of "prothrombin times" encountered in ordinary plasmas. In particular, they offer an attractive explanation of the frequently normal "Quick" test on newborn infants' blood, namely, that the very considerable *hyper*-proaccelerinemia of such cases(1) can *compensate* for the normally low levels, in infants, of prothrombin and proconvertin. There is no need, in our opinion, to postulate any obscure "convertibility" factor or some hypothetical "prothrombinogen."

Summary. In analysed systems representing artificially reconstituted plasmas of known composition with respect to prothrombin, (pro)convertin, and AcG, one-stage clotting-times with Ca-thromboplastin, essentially in the manner of the "prothrombin time" (Quick) test, show: (1) prothrombin concentration is a major variable. (2) (pro) convertin concentration is a minor variable, which, except at very low(10) levels, affects the clotting-times significantly only at very low prothrombin levels. (3) High (pro)convertin levels, short of an unexplained inhibitory phenomenon (minor), do not compensate significantly for low levels of prothrombin or of AcG. (4) AcG levels are a major variable. (5) Excessively high (200-300% of 'normal') AcG levels *can compensate* for moderately low prothrombin and proconvertin, causing the one-stage test to lose significance as a measure of these other two factors. (6) It seems valid to apply this last result to explain the normal "prothrombin time" (and lack of bleeding) in normal healthy newborn infants(1).

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Age Changes in Composition of Aorta of the Rabbit.* (22706)

Douglas Waugh, Arlene J. Maximchuk,† and James R. Stuart
(Introduced by G. L. Duff)
Department of Pathology, McGill University, Montreal, Canada

The important disabling and lethal vascular changes of ageing in man include those of atherosclerosis, calcification and hypertension. There has been considerable research, in man and in animals, on dietary induction of these conditions, and on alterations in blood chemistry that accompany their development (1). There are also reports on chemical alterations in vessels affected by atherosclerosis (2), calcification(3) and hypertension(4). However, there has been relatively little reference to age changes in chemical composition of vessels *not* affected by one or more of these conditions. Study of this aspect of vascular disease seems important, since the vessels of the young are relatively free of these damaging conditions. The present study was undertaken to determine some of the chemical phenomena of vascular ageing in the hope that the results might indicate some local factors that might render the vessels of the old more susceptible, if indeed they are, than those of the young. In view also of the rarity in ageing rabbits of spontaneous vascular disease equivalent to that in man, it was hoped that comparison of the findings in these two species might yield information bearing on the pathogenesis of the diseases noted above. It has been reported that in man the water content of the aortic wall either remains constant(3,5) or rises slightly(6) with increasing age. This is accompanied by progressive elevation of the total lipid(3,5) and of cholesterol(2,7). Calcium(3,5,8,9) and phosphorus (3) have also been reported as reaching higher concentrations in the human aorta with advancing age. Data on the sodium and potassium content of the human aorta have not come to our notice.

There appear to be no comparable data on age changes in the chemical composition of the rabbit aorta. Our preliminary studies showed that in ageing rabbits there is a significant and progressive rise in the water content of the aorta, associated with an even more striking fall in the quantity of combined aqueous and fat-soluble extractibles, (unpublished data). The present study was undertaken to confirm and amplify this observation.

Materials and methods. Forty-four female and 7 male New Zealand white rabbits were used. Because an adequate number of old males could not be obtained, the results tabulated and tested statistically are for the females only. There were 27 young animals, aged 2.5 to 3.5 months, and 24 old ones of 37 to 55 months. All were apparently healthy. Samples of blood were collected from a few animals (via the ear artery) several weeks before killing, and also from some immediately before killing. Some were subjected to blood pressure determinations by the non-traumatic method of Grant and Rothschild (10). All were fed Ogilvie Miracle Rabbit Pellets® and tap water *ad libitum*. The average period of acclimatization in our animal quarters after delivery from the dealer was one month. The environment was at normal indoor or "room temperature." The animals were killed by a blow on the back of the neck, and autopsies done immediately. The aorta from arch to renal arteries was removed, opened longitudinally, blotted free of gross blood with filter paper, and the adventitial fat dissected off as completely as possible. The specimen was transferred to a stoppered weighing bottle, placed in a desiccator, and weighed to 0.1 mg. The tissue was then lyophilized and the dry weight obtained. In transfer procedures the aorta was handled with forceps only. Sodium, potassium and calcium concentrations were determined using aortas from 18 young rabbits (all female) and 16 old ones (12 females, 4 males). Samples were pooled in 3's from young, and in pairs

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† Now at University Hospital, Edmonton, Alta.

TABLE I. Age Changes in Aortic Water of Rabbit.

No. of animals	Avg age, mo	Water, %
25	3	62.9 ± .4
19	46	65.4 ± .3

from old animals, to provide quantities large enough for accurate measurement. The pooled samples were ashed with nitric and perchloric acids. Sodium and potassium were determined by flame photometry, and calcium by the microadaptation of the Clark-Collip method(11). Data from female samples are designated in Table II as Group 1-a and 2-a. The aortas of the remaining 9 young (7 females, 2 males) and 8 old (7 female, 1 male) rabbits were used for determinations of total lipid, cholesterol and non-lipid phosphorus. These vessels were also tested for carbohydrates, reducing sugars and sulfur. Data from females are listed as groups 1-b and 2-b, Table III. After freeze-drying, the specimens were extracted in a Soxhlet apparatus, using 15 cc ethanol for a period of 24 hours, followed by cold ethyl ether overnight. The fat-free weight of the sample was recorded and the aorta was extracted in 15 cc double distilled water for an additional 24 hours. Following this the sample was desiccator-dried and weighed. The alcohol-ether extracts were evaporated and the residue taken up in petroleum ether. Total cholesterol content of this sample was then estimated by the Liebermann-Burchard reaction (12). The water extract was combined with the residue remaining after purification of the alcohol-ether extract and was tested for traces of carbohydrate, reducing sugars and sulfur. Molisch's reagent was used for carbohydrate, Benedict's quantitative reagent for reducing sugars and barium chloride solution for sulfur(13). The phosphorus content of the aqueous extract was estimated on duplicate aliquots using the Fiske-Subbarow technic (14).

Results. The qualitative tests for carbohydrate, reducing sugars and sulfur yielded negative results. Table I shows the mean values for water content of the aortas of 25 young and 19 older female animals. The aortas of the older animals contain an average of 2.5% more water than those of young animals, a small but highly significant difference ($p < .001$). Because of this change in water concentration, other values are reported per unit of dry weight.

In Table II are shown the aortic sodium, potassium and calcium concentrations. The potassium content remains unchanged, while there is a moderate but significant increment of sodium, and the calcium concentration is approximately doubled in the older animals ($p < .001$ for both Na and Ca). These changes are more marked than the increase in water concentration, and are significant even when related to the wet weight.

Table III compares the aortic content of lipid, cholesterol and phosphorus in the young and old animals. The results indicate a slightly but not significantly lower total lipid, and moderately higher levels of cholesterol ($p < .05$) and of phosphorus ($p < .02$) in the aortas of the older animals.

Discussion. These results confirm our pre-

TABLE II. Age Changes in Sodium, Potassium and Calcium of Aorta of Rabbit.

Group	No. of pooled samples	Age, mo	Na	K	Ca
			(mEq/100 g dry wt)		
1-a	6 (18)*	3	22.4 ± .4	11.3 ± .4	98 ± 9
2-a	6 (12)	46	28.6 ± 1.2	11.5 ± .3	219 ± 15

* Figures in parentheses show No. of aortas used.

TABLE III. Age Changes in Lipid, Cholesterol and Non-Lipid Phosphorus of Aorta of Rabbit.

Groups	No. of animals	Age, mo	Lipid, % dry wt	Cholesterol	Non-lipid phosphorus
				mg/100 g dry wt	
1-b	7	3	16.3 ± 1.7	463 ± 37	158 ± 12
2-b	7	45	13.7 ± 1.3	558 ± 22	210 ± 15

liminary finding of a higher aortic water concentration in old than in young rabbits although the increase found in this study, using freeze-drying, is less marked than that found previously by oven-drying at 110°C. Comparable studies in man have shown that aortic water either remains constant(3,5) with increasing age, or increases(6).

The rise in water content of the aorta associated with a rise in the sodium but constant potassium level might indicate that all or most of the added water was extracellular, *i.e.* that the aged aorta had become relatively oedematous. While this may well be so, there is some evidence to indicate that the change may be more complex. Other studies(15) have indicated age changes in the concentrations of sodium and potassium in rabbit erythrocytes, characterized by progressive replacement of intracellular potassium by sodium. If the same should be true of the cells of the aortic wall, then the extra water and sodium in the aortas of old animals could be both intra- and extracellular. Elevation of aortic water, sodium and potassium was found by Tobian and Binion(16) in hypertensive rats. The relation of the sodium increase to blood pressure in our animals is not clear. In general, adult rabbits have shown higher pressures than animals comparable in age to those of the present young group. However, other rabbits with a marked elevation of blood pressure due to bilateral renal compression did not have significantly altered aortic water, sodium or potassium concentration. A similar discrepancy was found for red cell electrolytes(15).

In the case of the other mineral constituents studied, calcium and phosphorus, our findings are in general agreement with those reported by others in man(3,5,8,9). In spite of the fact that in none of the aortas examined was there the slightest evidence of gross calcification, analysis showed a 100% increase of calcium in the older aortas. Phosphorus was increased by slightly more than 20%. The increment of phosphorus in the older animals is in about the same ratio to that of calcium as was found in man by Buck (3), *i.e.* 0.36:1. The calcium and phosphorus concentrations in the rabbit aorta were both considerably lower than Buck's lowest human values. This is curious because serum calcium levels in the rabbit are normally nearly double those in man(17).

A further difference between age changes in chemical constituents in the rabbit aorta and those in man is seen in the concentrations of total lipid and of cholesterol. For both these constituents the concentrations in the rabbit aorta are much lower than those reported in man(3,5,7). In addition, while the concentration of total lipid rises progressively with advancing age in man, our findings in the rabbit indicate no such increase, though there is an increase of doubtful significance ($p<.05$) in aortic cholesterol. It is worth noting that the only old male whose aortic cholesterol and total lipid were determined had values for both which were lower than those of any of the 7 old females studied.

In the interpretation of any age alterations in the rabbit one is handicapped by the lack of precise data on the natural longevity of rabbits as noted by Leary(18). On the basis of available information we have chosen to consider the two groups of animals in the present study as representing approximately childhood and middle-age.

Summary. A study of the aortas of 44 female rabbits in 2 age groups (3 mos. and 46 mos.) corresponding approximately to human childhood and middleage, indicates elevation of the water, sodium, calcium, non-lipid phosphorus, and possibly of the cholesterol content of the vessel wall in the older group. The potassium content remains relatively constant. The generally lower levels of total lipid and of cholesterol in the rabbit aorta compared with that of man probably account at least in part for the freedom of the former from spontaneous atherosclerosis.

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Serial Propagation of 3 Strains of Rabbit Fibroblasts; Their Susceptibility to Infection with Vaccinia Virus.* (22707)

R. F. HAFF† AND H. E. SWIM (Introduced by R. F. Parker)

Department of Microbiology, School of Medicine, Western Reserve University, Cleveland, O.

Tissue culture technics have been employed frequently for the propagation of viruses *in vitro*(1,2). There have been, however relatively few studies that attempt to elucidate in a simplified system which is potentially provided by tissue culture many fundamental problems regarding virus multiplication. To approach problems of this type in a definitive manner, it is necessary to have a continuous supply of uniform experimental material such as is provided by cultures of mammalian cells which can be propagated serially under controlled conditions. Cultures derived from different sources may vary considerably in their viral range and in their response to viral infection in a fashion analogous to laboratory animals. This indicates the need for cultures of more than one cell type as well as cells derived from a variety of animals. These studies were undertaken to investigate the possibility of establishing *in vitro*, strains of fibroblasts derived from rabbit tissues and to compare their relative susceptibilities to infection with vaccinia virus.

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† U. S. Public Health Service Research Fellow of The National Microbiological Institute.

TABLE I. Composition of Cell Culture Media.

Medium	Composition* (V/V)		
	%	%	%
CF	5 CEE	10 NHS	85 V-614
F7		10	90
F15	5 BEE	10	85
F17	10 CEE	20	70
F21	5	20	75
705	5	20	75 703

* The following abbreviations are employed: CEE = chick embryo extract; BEE = beef embryo extract; NHS = normal horse serum; V-614 = solution of Fischer *et al.*(5); 703 = solution 703 of Healy *et al.*(6).

Materials and methods. Media. The composition of the media employed for propagating cells is indicated in Table I. All media were sterilized by filtration through Selas filters of 02 porosity. Chick embryo extract (CEE) and beef embryo extract (BEE) were prepared as described previously(3). *Establishing cell cultures on glass.* Cell cultures were initiated in roller tubes according to the method of Swim and Parker(4). When growth in the roller tubes was well established, the cells were dislodged from the glass by treating with 0.02% trypsin (Nutritional Biochemicals Corp., 2 x crystallized—50% Mg SO_4) in V-614 solution(5) or in solution 703 (6), pH 7.9-8.3, for 10 minutes at 37°C. The

cells were then washed in nutrient fluid, the cell clumps largely dispersed by repeated pipetting. The cells were counted in a hemocytometer and 1.5 x 10^6 were added to T-60 flasks(7). The medium in each flask was replaced 3 times weekly. Subcultures were prepared by the use of trypsin. After treatment with trypsin, the cells were washed in nutrient fluid and added to fresh flasks (8 x 10^5 cells per T-60). *Vaccinia virus.* The CVII strain of vaccinia virus(8) which had been passed 1 to 3 times in cultures of rabbit-muscle fibroblasts was employed. *Titration of vaccinia virus in cell cultures.* One ml aliquots of a suspension containing 5 x 10^4 cells were added to 13 x 100 mm Pyrex tubes. Serial 10 fold dilutions of virus were prepared in solution V-614 and 0.1 ml of each dilution was added to a series of 5 tubes. The tubes were inclined at an angle to 5° and incubated at 37°C in a stationary position. After 24 hours, the tubes were transferred to a roller drum at 37°C. The virus titer (TC-ID_{50}) was determined on the basis of the lowest concentration of virus which destroyed the cells in 9 days by the method of Reed and Muench(9).

Results. Twenty-three strains of fibroblasts derived from tissues of the rabbit, and chick embryo were established *in vitro* and propagated serially in a variety of media. In spite of concentrated efforts to establish the conditions required for the continuous propagation of each strain of cells, only 3 proved to be capable of sustained growth in the media employed. The pedigrees of the successful strains are outlined in Fig. 1. It will be noted that the growth rate of each strain progressively declined after a period of active proliferation. This was accompanied at first by an increase in the number of granules in the cytoplasm of the cells; later, degenerating cells were observed and their numbers increased progressively until the bottoms of the flasks were covered with a dense layer of cellular debris (Fig. 2). Some of the cells continued to metabolize glucose, however, as indicated by the production of acid and fluid replacements were continued at regular intervals. During the 19th week a colony (about 1 mm in diameter) of cells, of normal appearance (Fig. 3), was observed in one of the four flasks in the series derived from adult rabbit testicle. The progeny of the cells from this colony (strain RT6-F17; Fig. 4) have continued to proliferate at a constant rate and are currently in the 57th passage.

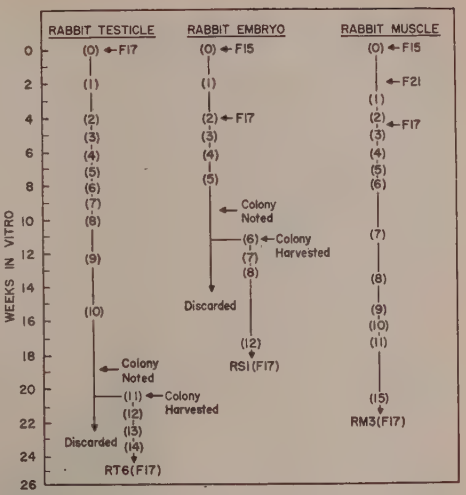


FIG. 1. Pedigrees of 3 strains of rabbit fibroblasts. Figures in parentheses refer to subculture number.

A second strain of fibroblasts was derived from the leg of a rabbit embryo of approximately 15 days' gestation (Fig. 1). During the 10th week three isolated colonies of normal appearing cells were observed in two of the five flasks in this series. The progeny of the cells from one of these colonies (Strain RS1-F17; Fig. 5) are now in the 38th passage.

A third strain of fibroblasts was derived from adult rabbit skeletal muscle. In this instance the degenerative changes which accompanied the decline in growth rate (Fig. 1) were not as extensive as those observed with strain RT6-F17 and RS1-F17. During the 13th week the overall appearance and growth rate of the culture began to improve gradually and after the 16th week it was possible to prepare subcultures at weekly intervals. This strain of fibroblasts, (RM3-F17; Fig. 6), continues to proliferate rapidly after 61 passages.

The other 20 cultures of fibroblasts which

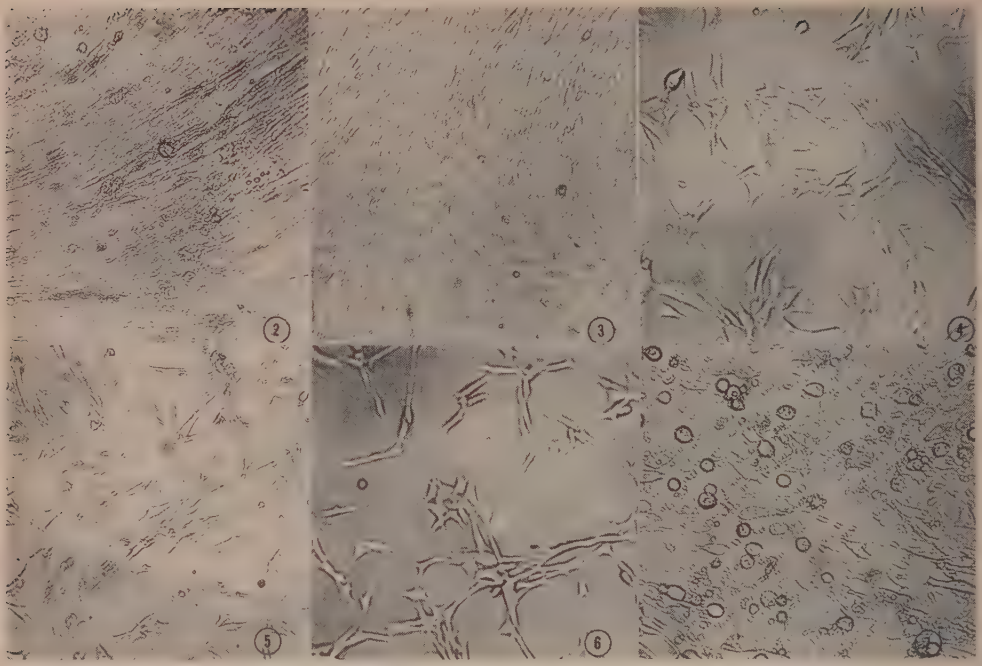


FIG. 2. Culture derived from rabbit testicle after 18 wk *in vitro*.
FIG. 3. Fibroblasts in original colony of RT6-F17 derived from culture illustrated in Fig. 2.
FIG. 4. Rabbit fibroblasts, strain RT6-F17 during 57th subculture.
FIG. 5. Rabbit fibroblasts, strain RS1-F17.
FIG. 6. Rabbit fibroblasts, strain RM3-F17.
FIG. 7. Culture of RM3-F17 three days after infection with 100 TC-ID_{50} of vaccinia virus.

were established (5 rabbit testicle; 2 each rabbit muscle, kidney, liver and lung; 7 chick-embryo heart or skeletal muscle) proliferated rapidly for 8-12 subcultures in a variety of media in addition to those indicated in Table I. After this period, the rate of growth either declined gradually over a period of several weeks or growth ceased abruptly. Numerous attempts to formulate a medium that would prolong the period of active growth of these cells have been unsuccessful. Each strain was maintained by periodic replacement of the medium until no viable or metabolizing cells appeared to be present in the flasks. Several cultures of chick embryo fibroblasts were maintained for 9 months without subculturing but failed to yield cells capable of continuous proliferation.

The following is a summary of the general nutritional requirements of the 3 strains of fibroblasts. Solution V-614 supplemented with CEE extract and NHS (medium F17, Table I) is an excellent medium for all 3 strains of fibroblasts. Media containing less NHS and CEE than F17 are adequate but the growth rate of cells is decreased proportionately whereas, media containing higher concentrations of NHS and CEE than F17 are inhibitory. The medium formulated by Eagle(10) for strain L will replace solution V-614 in F17 medium but the cells fail to proliferate when solution 199(11) or 703(6) is used. CEE is superior to BEE and extracts prepared from various adult rabbit organs are toxic. NHS can be replaced by bovine serum whereas human or rabbit serum is unsatisfactory.

Vaccinia virus multiplies in each of the 3 strains of fibroblasts and this is accompanied by the destruction of cells (Fig. 7). This cytopathogenic action afforded a method for comparing the susceptibility of cell strains to

TABLE II. Relative Susceptibilities of Cell Cultures to Infection with Vaccinia Virus.

Culture	Negative logarithm $TC\text{-}ID_{50}$/ml CF*	F15*	F7*
Lymphosarcoma (MB13)†	6.73	6.50	‡
Chick embryo§	6.08	5.50	‡
RM3 (F17)	5.82	5.12	4.68
RT6 "	5.55	5.55	4.84
RS1 "	5.50	5.38	4.44

* See Table I for composition of media; each value is avg for 4 or more titrations.

† Serially propagated in medium 705 as described previously(3).

‡ Cells degenerated in absence of virus.

§ Fibroblasts derived from skin-muscle of 10-day embryo; serially cultivated in CF; titrations performed at intervals from 3rd to 12th subculture.

vaccinia by employing them for titration of a standard pool of virus.

The relative susceptibilities of the 3 strains of rabbit fibroblasts are presented in Table II, together with results obtained with chick embryo fibroblasts and with lymphosarcoma cells, strain MB13. Strains RM3-F17, RT6-F17, RS1-F17 and chick embryo fibroblasts exhibit the same degree of susceptibility (medium CF) which is less than that observed for MB13 by a factor of approximately ten. The titer observed with MB13 is the same as that obtained in the embryonated egg by the pock method(12). When BEE is substituted for CEE in the medium, the relative susceptibilities of RT6-F17 and RS1-F17 are unchanged whereas, RM3-F17 appears to be less susceptible (compare media CF and F15). On the other hand, when embryo extract is omitted (medium F7) the 3 strains of rabbit cells are uniformly less susceptible. It is of interest that the susceptibility of RT6-F17 (subcultures 13-15; Fig. 1) which was derived from an isolated colony of cells is the same as the parent culture from which it was derived (subcultures 3-6; Fig. 1).

Discussion. The fact that all of the cells derived from normal tissue either ceased to proliferate or multiplied at a sharply reduced rate after an initial period of luxuriant growth is a perplexing problem which is not confined to fibroblasts from rabbit and chick embryo tissues‡(13). This phenomenon has received a minimum of attention primarily because in most instances cultures derived from normal tissues have not been propagated on glass for sufficient periods to observe this phenomenon. For example, certain strains of human fibroblasts cease to proliferate only after 30 subcultures *in vitro*.‡ One interpretation of the results of this study is that the usual tissue culture media are inadequate because they lack essential nutrients or because they are chronically toxic to most fibroblasts.

The results of this survey raise the question of the origin of the fibroblasts capable of continuous proliferation since only 3 of the 23 strains examined yielded such cells and because the latter appeared at a time when most of the cells in the individual cultures were either dead or degenerating. These data are subject to at least 2 interpretations: (1) fibroblasts capable of continuous proliferation were present in the original tissue inoculum and were preferentially selected by serial propagation; (2) in the course of serial propagation, a small proportion of the cells were altered sufficiently by mutation to be capable of sustained growth in F17. The feasibility of the latter is supported by studies of Sanford *et al.*(14) and by the fact that strains of human fibroblasts have been observed to alter their nutritional requirements on prolonged serial cultivation in response to changes in the composition of the medium.‡ Regardless of the mechanism with regard to the origin of the 3 strains of rabbit fibroblasts, it is clear that the proportion of cells derived from tissues of the rabbit and the chick embryo which are capable of sustained growth in the media employed is a very small fraction of the total. Furthermore, it appears that the isolation of permanent lines of fibroblasts from these sources as a routine procedure will require modifications in the composition of the usual tissue culture media.

Morphologically all 3 strains of rabbit fibroblasts are similar and each has maintained its characteristic appearance throughout the period *in vitro*. The most striking difference observed between strains RM3-F17, RT6-F17 and RS1-F17 is that the rate of growth of the former in F17 (12 fold per week) is about twice that of the other 2

‡ Unpublished experiments.

strains (Table II).

Summary. Twenty-three strains of fibroblasts derived from normal rabbit and chick embryo tissue were serially propagated on glass in a variety of media. All except 3 strains failed to proliferate for more than 8 to 12 subcultures. The 3 successful strains were derived from adult testis, embryonic skin-muscle and adult muscle respectively and continue to proliferate at a constant rate after 38 to 61 subcultures. Vaccinia virus multiplies in all 3 strains of fibroblasts resulting in the complete destruction of cells.

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Studies on Utilization of Sulfate Sulfur for Growth of the Chicken.*† (22708)

L. J. MACHLIN‡ AND P. B. PEARSON

Animal and Poultry Husbandry Research Branch, U. S. Department of Agriculture, Beltsville, Md.

It has been reported(1) that addition of sodium sulfate to a purified diet devoid of inorganic sulfur improved growth and feed efficiency of young chickens. Studies with S^{35}-sulfate have demonstrated that small amounts of sulfate sulfur are incorporated into cystine in the growing chicken(1,2) and laying hen (3), and larger portions into taurine by the chick embryo(4). In addition, the feathers of birds fed $Na_2S^{35}O_4$ contain a radioactive organic fraction which is not a sulfur amino acid and which has not been identified(1).

The purpose of these studies was to verify the growth response from sulfate sulfur, to determine whether such a response results from the conversion of sulfate sulfur to sulfur amino acids and to identify the radioactive compound appearing in the feathers after the feeding of $Na_2S^{35}O_4$.

Growth. The basal diet was a modification§ of that used by Gordon and Sizer(1), which contains 15% casein and 10% gelatin as the protein‖ source. Female New Hampshire X Silver Cornish crossbred chicks were fed the basal diet for 1 week. They were then weighed, divided into groups of 13 chicks of equal weight distribution and fed the experimental diets for 3 weeks. The chicks were reared under electrically heated brooders in wire-floored cages, in an air-conditioned room. Addition of 0.5% Na_2SO_4 to the basal

* This project was supported in part by the U. S. Atomic Energy Commission.

† An abstract of part of this work was published in *Poultry Sci.*, 1955, v34, 1209.

‡ Present address: Monsanto Chemical Co., St. Louis, Mo.

§ Wesson oil or lard was used as the fat. $MgCO_3$, $MnCl_2$ and copper acetate were used instead of the corresponding sulfates.

‖ The protein furnished about 0.54% methionine and 0.06% cystine. Since the estimate corresponds closely to the 0.132% total sulfur found by analysis the diet probably contained no inorganic sulfur.

TABLE I. Growth Response from Addition of 0.5% Na_2SO_4 to Low-Sulfur Diet.

Exp. No.	Basal diet Body wt (g) at 4 wk	0.5% Na_2SO_4	Increase, %	Level of significance*
1	160	232	45	.01
2	157	174	11	none
3	192	237	23	.01
	175	210	20	.05
4	285	300	5	none
5	204	283	39	.01
	224	276	23	.01
6	228	274	20	.05
7	239	294	23	.05
8	230	262	14	none
9	245	284	16	.05
Avg	213	257	22	

* Probability using Students' "t" test.

diet resulted in a growth response in every trial which was usually statistically significant (Table I). Feathering of birds fed the basal diet appeared normal.

Utilization of $Na_2S^{35}O_4$. A group of chicks was fed the basal diet plus 0.4% DL methionine. At 2 weeks of age the chicks were fed this diet plus 5 mg of $Na_2S^{35}O_4$ (1.6 mc) per kilo. One and 2 weeks after the addition of radioactive sulfate, 5 chicks were killed and the S^{35} in the sulfate, cystine, methionine, and taurine fractions determined for individual carcasses. The methods were essentially those previously described(5), except that paper chromatography was employed for further purification of taurine following chromatography on Dowex-50 resin. A summary of these results is given in Table II. Only trace amounts of radioactive cystine or methionine could be detected at 1 week following feeding of radioactive sulfate. After 2 weeks there was a measurable amount of radioactivity in these amino acids. Since only 1.5% of the S^{35} ingested was recovered in the cystine and methionine it would not appear that the rate of incorporation of sulfur into these amino acids was sufficient to significantly reduce the requirement for these amino acids in the diet. Taurine-S^{35} accounted for about 19% of the S^{35} recovered in the birds after 1 week of sulfate-S^{35} feeding and 24% after 2 weeks. This increase would suggest that taurine has a longer biological half-life than sulfate. A portion of the organic-S^{35} could not be accounted for as either cystine, methionine or taurine. It is likely that some of this S^{35} was present as unidentified compounds. These probably arise from the metabolism of taurine (6). Taurine accounted for an average of 6.7% and sulfate 5.6% of the total sulfur of the carcass of 4-week-old chickens.

TABLE II. Recovery of S^{35} from Compounds Isolated from Carcass of Chickens Fed $Na_2S^{35}O_4$.*

Compound	Duration of feeding period (wk)† 1	2
	% of total S^{35} recovered	
Sulfate	74.5	57.8
Cystine	trace	1.4
Methionine	"	.1
Taurine	18.9	24.1
Remainder	6.6	16.6
Total	100.0	100.0
% of dose‡ administered	22.7	19.7

* 1.6 mc/g of diet.
† Chickens were 2 wk of age at start of radioactive feeding period.
‡ Based on avg feed consumed.

Feather compound. The feathers from the birds fed sulfate-S^{35} were analyzed after acid hydrolysis. Sulfate, cystine and methionine accounted for only 47% of the S^{35} in these feathers. The remainder of the S^{35} behaved similarly to taurine on Dowex-50 columns. After paper chromatography, however, it did not give the same R_f as taurine with all solvent systems. A brown pigment was present in fractions containing the unknown S^{35} compound. In order to avoid any complication from this pigment in the colored feathers of the chicks used, a second study was conducted with White Leghorn chicks. In this study it was found that almost all of the unknown S^{35} could be extracted from the ground feathers with hot water. This water extract was subjected to chromatography on Dowex-50(4), followed by paper chromatography with 5 different solvent systems. Essentially all of the non-sulfate-S^{35} in the extract was thereby identified as taurine-S^{35}. Approximately 54 mg% of taurine (on dry matter basis) was found(4) in these feathers after acid hydrolysis. This accounted for about 10% of the free amino nitrogen and 0.6% of the total sul-

fur in the feathers.

A third study was conducted with New Hampshire X Silver Cornish birds. The S^{35} compound was freed of contamination with pigment by eluting it from large sheets of Whatman 3MM filter paper after development of radioactive fractions with n-propanol-1N NH_4OH (5:1). When this unknown S^{35} was rechromatogramed 90% of the S^{35} was found at the R_f values corresponding to taurine and the R_f was not changed after treatment with H_2O_2. With some chromatograms a small second peak of S^{35} was detected suggesting the presence of another sulfur compound. However, most of the unknown S^{35} was apparently taurine whose detection was obscured by the presence of feather pigment.

The significance of taurine in feathers is not known. It has been suggested(6) that end-products of metabolism such as uric acid and creatinine are "trapped" in the feather during the process of rapid growth and keratinization. Similar reasoning could be applied to taurine.

Discussion. Our work confirms the report of Gordon and Sizer(1) on the ability of sodium sulfate to stimulate the growth of the chicken, but differs with their observations on feathering. These workers reported that feathering of chickens fed a low-sulfur diet was poor and was improved by addition of sulfate. In our experience, the feather development of chickens fed a low-sulfur diet was satisfactory and showed no improvement when sulfate was added to the diet. Sulfate sulfur was not utilized for synthesis of sulfur amino acids in amounts large enough to suggest nutritional significance, but was incorporated into tissues chiefly as protein-bound sulfate. The growth response from feeding sulfate therefore may be due to a physiological requirement for sulfate *per se*. This requirement can evidently be satisfied by dietary sulfate or by sulfate furnished by the oxidation of the sulfur amino acids. Thus sulfate may "spare" the methionine or cystine which would be oxidized to provide the sulfate necessary for the growth of the chicken. The sulfur amino acid requirements have been determined with basal diets containing varying amounts of sulfate sulfur. Therefore, on the basis of data now available, it is impossible to estimate to what extent sulfate sulfur can replace methionine or cystine sulfur. After feeding of $Na_2S^{35}O_4$, over 20% of the S^{35} was recovered in the chickens as taurine and what are probably taurine metabolites. Taurine accounts for a substantial part (6.7%) of the total sulfur in the chicken and it is quite widely distributed in the tissues. It is possible that free taurine has some function other than as component of bile acids(6) and therefore dietary sulfate might also be of value as a precursor of taurine.

Summary. 1. Addition of 0.5% sodium sulfate to a diet low in sulfur stimulated growth of the young chicken. 2. When $Na_2S^{35}O_4$ was fed for a period of 1 week, approximately 23% of the S^{35} ingested was incorporated into the tissues. Most of the S^{35} was recovered as sulfate-S^{35}. However, about 19% was present as taurine-S^{35}, 6.6% as unknown organic compounds and barely detectable amounts as sulfur amino acids. Two weeks after $Na_2S^{35}O_4$ feeding a larger proportion of S^{35} was present in the carcasses as taurine and unidentified organic compounds and 1.5% of the S^{35} in the chickens was accounted for as cystine and methionine. It was concluded that there is a physiological requirement for sulfate *per se* and that dietary sulfate promotes growth primarily by providing sulfur for this purpose. 3. Taurine was found to be the major sulfur constituent of the water-soluble portion of feathers.

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Cultivation of L Strain (Earle) Mouse Cells in Bacteriological Media Combined with Horse Serum. (22709)

Sami A. Mayyasi and David M. Schuurmans. (Introduced by Dorothy G. Smith)

U. S. Army, Chemical Corps, Fort Detrick, Frederick, Md.

Research in the development of tissue culture media has been divided into two major areas. The first is the attempt to develop completely chemically defined media(1-8). The second area of research is the attempt to substitute various processed materials for blood serum and tissue extracts currently used in many tissue culture media. These processed materials, which include yeast extract(9), peptone preparations(10-13) and serum fractions(14,15), are sometimes combined with natural body substances(16) or with chemically defined media(1,3,4,17). This paper reports the development of a tissue culture medium which incorporates commercially available bacteriological preparations as supplements to blood serum in the cultivation of Earle's L strain of mouse fibroblasts.

Materials and methods. L cells(18) grown at 37°C for 3 to 5 days were scraped from T-60 Earle flasks and washed twice with Hanks' balanced salt solution (BSS). The cells were suspended in BSS and added to the various media which were then dispensed in 1 ml aliquots to Porter flasks. The concentration of cells in the Porter flasks ranged from 100,000 to 125,000 cells per ml. The following preparations have been tested for stimulation of L cell growth: Bacto Yeast Extract, Bacto Beef Extract, Bacto Casamino Acids, Difco Proteose Peptone, Difco Proteose Peptone #2, Difco Proteose Peptone #3, Difco Neopeptone, Tryptone, Tryptose, and Heart Infusion Broth, all produced by Difco Laboratories; Phytone, and Trypticase, produced by the Baltimore Biological Laboratory and N-Z Case produced by the Sheffield Chemical Co. The preparations to be screened were prepared as a 2% solution in BSS and sterilized by filtration through fritted glass. Concentrations of 2, 1, 0.1, and 0.01% were generally tested. In addition, these concentrations in BSS were tested in combination with 10% horse serum. The standard reference medium consisted of 60% medium 199,* 20% whole egg ultrafiltrate,* and 20% horse serum. Fifty units each of penicillin and streptomycin were added to each flask. All cultures were prepared in triplicate and incubated at 37°C. Various qualitative estimates were made to evaluate the growth rate and the appearance of cells from day to day. In addition to these qualitative estimates, quantitative determinations were made by taking total cell counts on the fourth day. In other short term experiments designed to test for stimulation of growth, a daily count was made for 5 days. The total cell count was made by removing the medium and replacing it with 1 ml of 0.25% trypsin in saline. Cell removal was hastened by shaking the Porter flasks on a New Brunswick rotary-type shaker operating at 100 rpm. The cells thus removed were counted in a hemocytometer. Two total cell counts were done on each Porter flask. The values shown in the figure and in the table represent the average count of triplicate flasks of a single experiment. The results of this experiment are representative of several that were performed. Preparations that showed some enhancement of growth were combined and tested in the presence of various concentrations of horse serum.

Results. Among the products screened, yeast extract and proteose peptone #3 gave the best results. One tenth % yeast extract was found to be the optimum concentration for proliferation of L cells in the presence of horse serum. This agrees with the results of Robertson with Hela cells(9). The optimum concentration of proteose peptone #3 was found to be 0.1%. No multiplication occurred in the absence of horse serum. The growth of L cells in a medium consisting of 0.1% yeast extract and 0.1% proteose pep-

* Obtained from Microbiological Associates, Bethesda, Md.

TABLE I. Multiplication of L Cells in Difco Bacto-Yeast Extract, Proteose Peptone #3, and Horse Serum.*

% yeast extract	% proteose peptone	% horse serum	Total cell count on 4th day $\times 10^5$	Fold increase
.1	0	20	6.00	4.8
.1	0	10	3.44	2.8
.1	0	2.5	1.52	1.2
0	.1	20	4.49	3.6
0	.1	10	3.29	2.7
0	.1	2.5	1.35	1.1
.1	.1	20	6.63	5.3
.1	.1	10	4.99	4.0
.1	.1	5	2.29	1.8
.1	.1	2.5	1.98	1.6
.1	.1	0	.46	.4
Standard reference medium†			3.74	3.0

* Initial inoculum: 1.25×10^5 cells/ml.
† 60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum.

tone #3 in the presence of various concentrations of horse serum is presented in Table I. The same table shows the growth obtained when these constituents were tested separately in the presence of horse serum. The growth of cells in 0.1% yeast extract or 0.1% proteose peptone #3 in the presence of 20% horse serum exceeded growth obtained in the standard reference medium. Growth in the presence of 10% horse serum was also better than that of the standard reference medium.

Among the combinations tried, 0.1% yeast extract combined with 0.1% proteose peptone #3 in the presence of horse serum gave maximum growth and exceeded that obtained using either alone. This was true at all levels of horse serum tested. There was a 5.3 and 4.0 fold increase in the total cell count in 4 days at the 20 and 10% serum levels respectively, compared to 3.0 fold increase in the standard reference medium. Although the total cell count at serum levels below 10% did not reach that of the standard reference medium, a significant degree of cell multiplication occurred at these low serum levels. There was a 1.8 and 1.6 fold increase in the total cell count at the 5 and 2.5% levels of horse serum.

Fig. 1 shows the growth curve of L cells in these two nutrients when combined with various concentrations of horse serum. The growth rate of L cells in yeast extract and proteose peptone #3 combined with 20 and 10% horse serum exceeded the rate obtained in the standard reference medium, after 5 days incubation.

Fig. 2-4 show photographs of the appearance of L cells grown in 0.1% yeast extract and 0.1% proteose peptone #3 combined with various concentrations of horse serum. The cells are thinner and more sparse in cultures where serum has been omitted.

In preliminary experiments L cells have also been cultivated for 11 days in the standard reference medium and in the yeast extract-proteose peptone #3 combination with horse serum levels of 20, 10 and 5%. The medium was changed twice during this period and the cells were transferred from T-60 Earle flasks to Roux bottles after the seventh day of incubation. The total cell count of these cultures after 11 days incubation had increased 43.7, 47.4, 34.5, and 29.2 fold re-

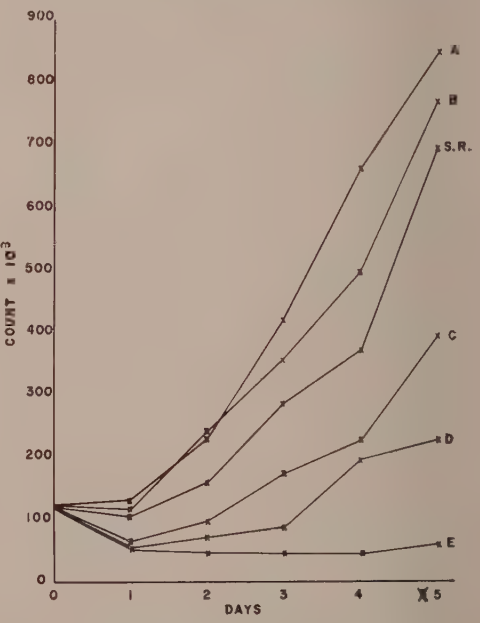


FIG. 1. Growth of L cells in yeast extract (y. e.) and proteose peptone #3 (p-p #3) combined with various concentrations of horse serum (HoS).

A. 0.1% y. e. and 0.1% p-p #3 plus 20 % HoS
B. 10 %
C. 5 %
D. 2.5%
E. 0 %

S. R. Stand. reference medium (60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum)



FIG. 2 (left). Earle's L strain cells from the mouse after 4 days in the standard reference medium (60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum). 100×.

FIG. 3 (center). L cells after 4 days in 0.1% Bacto-yeast extract and 0.1% proteose peptone #3, Difco, with 20% horse serum. Growth with 10% horse serum was equally good. 100×.

FIG. 4 (right). L cells after 4 days in 0.1% Bacto-yeast extract and 0.1% proteose peptone #3, without horse serum. 100×.

spectively. Under these conditions, the yeast extract-proteose peptone #3 combination in the presence of 20% horse serum produced slightly more growth than did the standard reference medium.

The medium reported here supported the growth of Gey's MBIII strain (mouse lymphoblasts) (19), Maben strain (a human epithelium-like cell derived from an adenocarcinoma of the lung) (20), and a strain of guinea pig lung fibroblasts that was isolated in this laboratory. Eighteen serial passages of L cells have been made in this medium containing 10% horse serum during a period of 17 weeks. The MBIII strain, Maben strain, and the guinea pig lung fibroblasts have been subcultured 13, 5, and 13 times during a 12, 6, and 18 week period respectively. The growth of these cell lines is undiminished and their appearance is normal.

The yeast extract-proteose peptone #3 medium, without horse serum, can be prepared as one solution and autoclaved.

Discussion. The present findings are consistent with the earlier reports that yeast extract and peptones support the proliferation of cells when combined with serum. The rapid multiplication of washed cells in the yeast extract-proteose peptone #3 medium reported here suggests that this medium contains growth factors that supplement blood serum and promote the growth of several lines of mammalian cells. Yeast extract is known to be an excellent source of B vitamins, and peptones are a rich source of nitrogenous compounds. The possibility that other unidentified growth factors are present in these preparations can not be ruled out.

It is significant to point out that the growth obtained in this medium, combined with horse serum, was slightly better than the growth obtained in a synthetic medium supplemented with whole egg ultrafiltrate and horse serum. The medium described here contains commercially available products that are cheap, reproducible, and convenient to handle. Their use in the large-scale cultivation of cells is therefore advantageous.

Summary. The combination of 0.1% yeast extract and 0.1% proteose peptone #3 in Hanks' balanced salt solution plus 20% horse serum stimulated the rate of growth and produced a higher total count of L cells at the end of 5 days than did the standard reference

medium (60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum). Lowering the serum content in the new medium to 10% still permitted better growth than the standard reference medium. The yeast extract-proteose peptone solution could be sterilized by autoclaving without affecting its growth-stimulating properties.

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Evaluation of a Rotating Disc Type Reservoir-Oxygenator.* (22710)

FREDERICK S. CROSS, ROBERT M. BERNE, YOICHI HIROSE, RICHARD D. JONES, AND EARLE B. KAY

Surgical Research Laboratory, St. Luke's Hospital, Cleveland, O.

The historical development of various pump-oxygenators is well covered in the literature(1,2,3). Many of the early machines, utilized both experimentally and clinically, bypassed but one side of the heart, this being a simpler solution to the overall problem(4, 5,6). However, Gibbon(7) and Dennis(8) have continually stressed the necessity of total cardiac bypass as a requirement for an entirely satisfactory pump-oxygenator. Lillehei and associates(9,10,11,12) hastened the reality of direct vision intracardiac surgery through the use of controlled cross-circulation, oxygenated blood reservoirs, and biological oxygenators, as temporary expedients before the final utilization of a pump-oxygenator. They also have stressed the concept of the low flow principle which has been satisfactory in their hands for shorter periods of bypass, and which possibly has simplified certain problems inherent in the development of effective pump-oxygenators.

It was the purpose of the present study to evaluate a rotating disc type reservoir-oxygenator, which has been used successfully for open intracardiac surgery experimentally and clinically.

Description of apparatus. The essential components of the present pump-oxygenator are a model TS Sigmamotor pump and a rotating disc type reservoir-oxygenator. (Fig. 1 and 2). The basic mechanism of the oxygenator is a series of 59 plastic coated stain-

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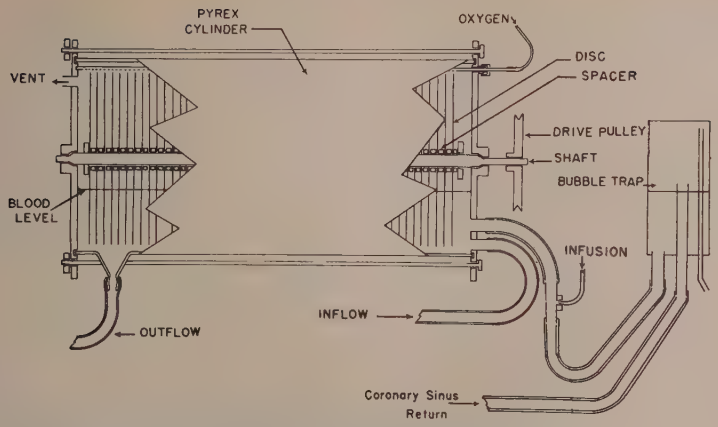


FIG. 1. Diagram of reservoir-oxygenator with attached defoaming chamber for coronary sinus return.

less steel discs 0.15 inch (0.4 mm) thick, and 4 13/16 inches (12.2 cm) in diameter, mounted 0.18 inch (0.5 mm) apart by means of stainless steel spacers on a central shaft. Previously the stainless steel discs were coated with silicone resin; at present they are coated with teflon. The disc assembly is supported horizontally within a silicone-coated pyrex cylinder 13 inches (33 cm) long, and 5¼ inches (13.3 cm) in diameter, by gasketed end plates of stainless steel, likewise treated with silicone resin. Blood is introduced at one end of the cylinder and is removed from the bottom of the opposite end. Rotation of the discs effectively prevents channeling of the blood along the bottom of

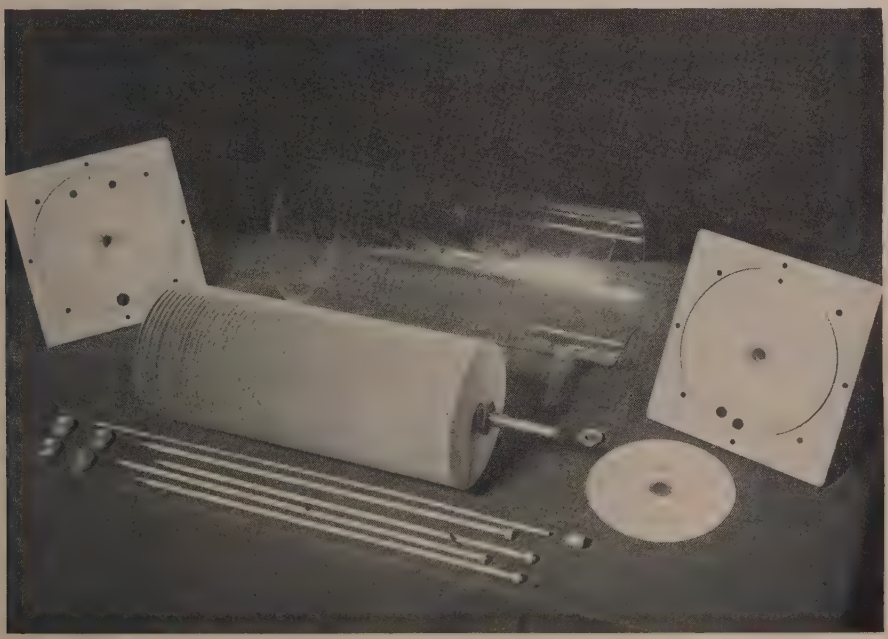


FIG. 2. Reservoir-oxygenator dissembled to show component parts.

the cylinder. The oxygenator is primed with 1400 cc of whole blood to immerse the discs to 1⅝ inches (4.1 cm), a level calculated to provide maximum exposure of blood to oxygen. When the discs rotate the blood films on the peripheral 1⅝ inches of each disc for exposure to oxygen. With the oxygenator primed with 1400 cc the static exposed area (that above the blood level) of the 59 discs is 9 square feet or 0.84 square meter. At a disc rate of 120 revolutions per minute, the rate generally employed, 1080 square feet or 110 square meters of filmed blood is exposed to oxygen per minute. To turn the discs, a pulley mounted on one end of the shaft is driven through a V-belt by a 1/80 horsepower motor controlled by a variac power rheostat. Oxygen is supplied at a rate of 5 liters per minute through a perforated stainless steel tube mounted within the cylinder above the discs. The oxygen is warmed by passing through tubing immersed in a sand-bath, heated by an electric mantle. The oxygenator itself is warmed by a wrapping of silicon rubber-covered resistance wire, the temperature of which is controlled by a variac. All electrical connections are mounted above the oxygenator, over 5 feet above the floor, to eliminate explosion hazards. Heat may be supplied selectively by increasing the current through the resistance wire and condensation within the oxygenator has been effectively prevented. Provision is made at the inlet face of the oxygenator for filling prior to use, the addition of blood to the cylinder during a run, and for sampling the venous return to the oxygenator from the patient. The output face is provided with openings to vent off the circulating oxygen, for collection of samples of oxygenated blood, and to receive a thermocouple for continuous monitoring of blood temperature. Recently a sump type suction tip has been used to collect coronary sinus return from the right atrium, utilizing a second Sigmamotor pump. Coronary sinus blood is pumped first to a defoaming chamber, from which it is returned to the input end of the oxygenating cylinder. Venous cannulations have been made with expendable nasal oxygen catheters, or with specially constructed adaptors of polyethylene, and all external tubing is tygon, 5/16 inch internal diameter, except the short sections of silicone coated rubber tubes in the pump head. To reduce turbulence and fibrin formation, all the adaptors are of high tensile, polished polyethylene, of the same internal diameter as the tubing they receive. A bubble trap has not been necessary on the outflow line since there is no bubbling present in the oxygenator, and filters in the external circuit are not used. Pump speeds are regulated through a previously calibrated speed changer. The oxygenator can easily be dissembled into its component parts for cleaning. It is sterilized in the autoclave after reassembly including the oxygen flow tube and the heating wire. The inside is then dried by flushing with oxygen or air while it is still hot, or by standing in the hot autoclave. Connecting tubes are sterilized in Zepharin for twenty-four hours and then rinsed and mounted in the pump circuit. The tubing is then rinsed in two liters of saline solution before being connected to the patient.

Method of study. Effective pump-oxygenators must maintain circulation as well as oxygenation in total cardiac bypass with a low morbidity and mortality rate. To evaluate the present pump-oxygenator, both acute and survival experiments were carried out with dogs, as well as studies utilizing cow blood. Observations carried out during the cardiac bypass were continuous mean blood pressure recordings, blood oxygen and carbon dioxide content, blood pH, plasma hemoglobin, hematocrit, and platelet and red and white blood cell counts. It was difficult to evaluate the oxygenating capacity of the oxygenator by dog experiments alone because of limitations imposed by the size of the available dogs. For this reason, experiments were done using cow blood. In these experiments, warmed desaturated cow blood was run through the oxygenator at the different flow rates to be studied. In each experiment, after 4 liters of blood had passed through the oxygenator and equilibrium had been established, samples of venous and oxygenated blood were taken and analyzed for their oxygen content. Blood samples for oxygen determination were packed in ice and the oxygen content was de-

termined in duplicate within 24 hours by the method of Roughton and Scholander.

Results. Mortality and morbidity were necessarily high in the developmental stage of the pump-oxygenator. However, after the basic design and technics were standardized a survival series of 20 dogs was carried out in which there were 2 deaths. In 11 of the dogs, simple cardiac bypass without cardiotomy was done, with one death due to undetermined causes; and in the remaining 9 dogs a ventricular cardiotomy was done with one death due to postoperative ventricular fibrillation following the institution of a citrated blood transfusion.

Perfusion pressures measured as mean arterial blood pressures with a mercury manometer, at flow rates from 40 to 80 cc/kg/min., averaged from 50 to 80 mm Hg. To maintain such pressures it was necessary in most experiments to add varying amounts of blood to the total circulating blood volume even though there was no demonstrable extrinsic loss. This could be partly compensated for by adding small amounts of neosynephrine to the oxygenator. Although the blood pressure could be affected in some measure by increased perfusion rates, this was not constant, and initial small rises were not maintained.

A specific value as to the oxygenating capacity and efficiency of the oxygenator cannot be given at present. With such factors as oxygen flow rate, disc rate, and blood temperature held constant, the efficiency of the oxygenator varied not only with the blood flow rate but also with the degree of desaturation of the venous blood returning to the oxygenator. With low oxygen saturations in the venous blood the oxygenator was more efficient in that more cubic centimeters of oxygen were added to the blood per minute; but lower arterial oxygen saturations were obtained. The reverse was true with higher levels of venous oxygen saturation. This is well illustrated in experiments 8 and 9, 16 and 17, 24 and 25, in Table I. In these experiments the efficiency of the oxygenator ranged from 67 cc/min. of oxygen added to the circulating blood, to 207 cc of oxygen added per minute. The arteriovenous oxygen difference ranged from 3.7 to 13 vol. %, depending on the oxygen content of the venous blood and the blood flow rate employed. Pertinent oxygenation data from a typical dog perfusion experiment are found in Table II. There was adequate oxygenation at a flow rate of 2000 cc of blood per minute (59 cc/kg body weight per minute) with an arteriovenous oxygen difference of 6.8 vol. % and an oxygen uptake of 136 cc per minute.

Hemolysis was not excessive (Table III). The increase in serum hemoglobin over a 30 minute pumping period ranged from 10 mg% to 160 mg%. Variations in serum hemoglobin could not be related to such factors as disc speeds below 120 revolutions per minute, or pump speeds, but the degree of

TABLE I. 25 Oxygenation Experiments Using Cow Blood.

Exp. No.	Blood flow, cc/min.	Oxygen saturation vol, % Arterial	Venous	A-V diff.	cc O_2/min. added to blood
1	660	20.3	7.4	12.9	85
2	940	18.9	11.8	7.1	67
3	1060	17.6	5.0	12.5	128
4	1125	17.6	5.3	12.3	138
5	1200	14.4	1.4	13.0	156
6	1224	15.9	4.8	11.1	136
7	1240	19.1	7.4	11.7	145
8	1330	15.0	4.8	10.2	149
9	1350	17.5	11.7	5.8	78
10	1475	15.4	7.4	8.0	118
11	1500	12.3	1.9	10.4	156
12	1580	15.5	5.3	10.2	138
13	1670	15.6	5.0	10.6	177
14	1710	14.9	5.1	9.8	168
15	1920	14.7	7.6	7.1	136
16	2060	13.8	5.5	8.3	171
17	2120	15.1	11.4	3.7	98
18	2250	13.7	4.8	8.9	203
19	2500	13.2	5.1	8.1	203
20	2537	10.5	5.5	5.0	127
21	2600	10.4	3.3	7.1	182
22	2640	12.6	7.6	5.0	132
23	2860	15.8	11.8	4.0	114
24	3080	11.8	5.1	6.7	207
25	3080	16.1	11.8	4.3	132

TABLE II. Typical Dog Cardiac Bypass Experiment.

Wt, kg	Blood flow, cc/min.	Disc rate, r.p.m.	O_2 saturation vol, % Arterial	Venous	cc O_2/min. added to blood
34	1000	120	18.3	10.3	80
	1600	120	18.9	10.1	141
	2000*	120	18.6	11.8	136

* 59 cc/kg/min.

TABLE III. Average Increase in Serum Hemoglobin.

	Interval of bypass			
	10 min.	20 min.	30 min.	40 min.
No. of exp.	17	18	14	4
Serum hemoglobin (mg/100 cc)	29	36.4	67.7	88.5

hemolysis did increase with pumping time. At disc rates in excess of 120 revolutions per minute hemolysis was increased by the added churning, and in addition foaming occurred in the oxygenator. There was moderate destruction of platelets. In 13 experiments in which they were counted, the platelets dropped from an average of 184,770 to 96,000, or roughly to one-half of their preperfusion value over a period of 30 minutes. When protamine sulphate was given in amounts equal to one-half the calculated dose of heparin, and fresh citrated blood given postoperatively, the clotting time was restored to normal. Postoperative bleeding was not a serious complication. Leukocyte counts showed no consistent change following periods of cardiac bypass. Blood carbon dioxide content and pH studies are better controlled in the clinical cases done to date and will be discussed later.

Discussion. The oxygenator described is simple in design, easy to operate, and can be constructed to oxygenate over a wide range of blood flow rates without requiring excessive blood for priming purposes. Although it resembles the Bjork(13) apparatus in general principles, it differs in certain specific aspects of its design. More plates have been used and they are teflon coated which appears to reduce the degree of hemolysis and eliminates the tendency for fibrin formation on the disc surfaces. By increasing the number of discs and filling the cylinder to a higher level, the minute surface area of exposure to oxygen is 110 square meters compared to 44 square meters for the small oxygenator and the somewhat higher value for the large oxygenator of Bjork. The pump-oxygenator here described is considered to be the basic experimental model and it has been undergoing continual modifications which retain the basic principles of construction described. The oxygenating reservoirs are now being constructed from plexiglass and the discs are being made from teflon rather than from the stainless steel coated with teflon. In addition, a large cylinder is now available to increase the oxygenating capacity of the unit for use in adults; and a smaller cylinder is being constructed for use with infants in order to reduce the necessary priming volume. The present unit has been used in 30 clinical cases to date with no deaths specifically attributable to the pump-oxygenator. Details of this experience will be published later.

Summary. 1. A rotating disc type reservoir-oxygenator is described. 2. Its effectiveness in maintaining circulation and oxygenation is discussed. 3. It has proven satisfactory in both its laboratory and clinical application.

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Lack of Effect of Carbutamide on Activity of Rat Liver Glucose-6-Phosphatase. (22711)

CARL A. KUETHER, MARGUERITE R. CLARK, EARL G. SCOTT, HENRY M. LEE, AND C. W. PETTINGA

Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

Carbutamide (BZ-55, or p-aminophenylsulfonyl butyl carbamide) exhibits hypoglycemic activity in normal animals and humans and in some diabetic individuals(1,2,3). One possible explanation of this physiological activity is that carbutamide interferes with the conversion of glycogen to glucose by inhibiting one of the enzyme systems involved in this conversion. The observation of Miller and Dulin(4) that liver glycogen levels are elevated after the administration of 1-butyl-3-p-tolylsulfonyl urea, an analog of carbutamide, appears to support this explanation.

Glucose-6-phosphatase, one of the enzymes involved in the conversion of glycogen to glucose, is more active in the liver of the diabetic animal(5,6), and is less active in the presence of glycogen storage disease(7). This paper describes a study on the effect of carbutamide on glucose-6-phosphatase activity of rat liver *in vitro,* and correlates these findings with the levels of carbutamide which were found in liver and plasma of animals treated with the drug.

Materials and methods. Since it has been shown by deDuve(8) and Langdon *et al.*(6) that most of the glucose-6-phosphatase activity is found in the microsomal fraction of liver homogenates, it was decided to use these sub-cellular particulate fractions as the enzyme source. Therefore active microsomal suspensions were prepared from the livers of normal rats by a method similar to that described by Langdon. These were kept frozen until used. In this condition no decrease in glucose-6-phosphatase activity was observed over a period of several weeks. The reaction mixture consisted of 0.02 *M* substrate, either the sodium salt of glucose-6-phosphate or β-glycerol phosphate, .02 *M* maleate buffer, pH 6.5, carbutamide or ammonium molybdate solution in concentrations indicated elsewhere, and the microsomal suspension derived from 40 mg of liver and containing about 0.2 mg of nitrogen. The total volume was 0.5 ml and the final pH 6.5. Carbutamide was dissolved in NaOH solution and adjusted to pH 8.0 before addition to the reaction mixture. Phosphate was determined according to Fiske and SubbaRow(9). The enzymatic activity was indicated by percent of total substrate hydrolyzed. The hydrolysis in the presence of inhibitors was compared with that of control experiments for which the activity was expressed as 100. Following the administration of various amounts of carbutamide to normal fasted rats, the concentrations in liver and plasma were estimated by the method of Bratton and Marshall(10). Blood sugar levels were determined by the method of Haslewood and Strookman(11) immediately before treatment and just before sacrifice. To gain some idea of the extent of binding of carbutamide by plasma proteins, a plasma ultrafiltration method similar to that described by Coolidge(12) was used. Fractionation of the livers of treated rats into various subcellular components was made as described by Umbreit, Burris, and Stauffer(13).

Results. The enzyme preparation was characterized by determining the effect of substrate and enzyme concentration on the extent of the reaction, by utilizing a known inhibitor of the enzyme, and by using a substrate not readily hydrolyzed by glucose-6-phosphatase. The velocity of the reaction was directly proportional to the enzyme concentration. The reaction was linear for the first 20 minutes, after which time the rate decreased quite rapidly.

For the inhibition studies, an incubation time of 15 minutes was utilized to eliminate variations caused by inactivation of the enzyme(14). The substrate concentration of 2×10^{-2} *M* served to minimize changes in substrate concentration during the course of the enzymatic reaction. Thus, in the inhibition studies the reaction followed zero order kin-

TABLE I. Effect of Carbutamide and Molybdate on Glucose-6-Phosphatase Activity of Rat Liver Microsomes.

Addition, moles/l	% of substrate hydrolyzed 1	2	3	4	Mean	Relative activity
None	25.8	25.7	25.0	26.8	25.8	100
Carbutamide 10^{-5}	31.2	28.0	31.5		30.5	118
10^{-4}	26.6	27.4	27.2		27.1	105
10^{-3}	25.5	26.7	28.1		26.8	104
10^{-2}		22.6	22.9		22.8	88
Ammonium molybdate 10^{-4}				16.2		63
10^{-3}				2.3		9
3.2×10^{-3}				0		0
10^{-2}				0		0

TABLE II. Distribution of Carbutamide in Plasma and Liver of Rats 4 Hours after Oral Administration.

Tissue	Dosage of carbutamide, mg/kg 1000	500	200	100†	50	25
Plasma, μg/ml	1,165	676	330	250	95	73
Plasma ultrafiltrate, μg/ml	850	460	205	93	31	21–31
Liver, μg/g	665	358	137	94	66	35
Liver water, μg/ml*	900	485	185	127	89	47
Blood sugar change, mg %	−15.5	−21.0	−17.5	−5.2	−14	+13.5

* Calculated from an assumed moisture content for liver of 74%.
† Avg of 4 rats; all others on single rat experiments.

etics. The enzyme preparation was relatively specific for glucose-6-phosphate as substrate. Under identical conditions the hydrolysis of glucose-6-phosphate was 25 times the rate of hydrolysis of sodium β-glycerol phosphate.

Addition of carbutamide in concentrations from 10^{-5} to 10^{-2} *M* (0.27 to 270 mg per 100 ml) had no significant effect on the activity of the enzyme (Table I). In contrast, ammonium molybdate, a known inhibitor of glucose-6-phosphatase(15), produced complete inhibition of the enzyme at a concentration of 3.2×10^{-3} *M*.

In Tables II and III are presented data on concentrations of carbutamide in plasma and liver after administration of the drug in doses of 25 to 1000 mg per kg orally. Plasma concentrations were higher than the concentrations in liver. When the drug was administered in doses greater than 100 mg per kg there was a fair correlation between the concentration of free carbutamide in plasma and the amounts of the drug dissolved in the water content of the liver. The latter was calculated by dividing the observed concentrations of carbutamide per g wet weight of liver by an assumed moisture content of the tissue of 74%. The concentrations observed in these *in vivo* distribution studies were all within the limits of the concentrations tested with the *in vitro* glucose-6-phosphatase assay, *i.e.* 3.3×10^{-3} to 2.4×10^{-4} *M*. Very little if any of the carbutamide found in the liver was bound to any particulate fraction.

TABLE III. Distribution of Carbutamide in Rat Liver Intracellular Fractions. Animals received 100 mg/kg orally; sacrificed 4 hr after treatment.

Fraction	Exp. 1 (μg/g liver)	Exp. 2 (μg/g liver)
Whole homogenate	133.0	97.0
Nuclear	5.0	2.5
Mitochondrial	4.3	0.7
Microsomal	2.5	2.0
Supernatant	100.7	104.0

Discussion. Although it seemed reasonable to postulate that carbutamide might exert its hypoglycemic activity by inhibiting glucose-6-phosphatase, the data presented here demonstrate that carbutamide had no significant effect on rat liver microsome preparations of glucose-6-phosphatase in a range of concentrations including those found to be effective in lowering blood sugar. Also the data show that very little of the drug was bound to the liver intracellular particles. Therefore the

hypoglycemic activity of carbutamide must be explained on some other basis than inhibition of glucose-6-phosphatase.

Vaughan(16) has reported a similar lack of effect of 1-butyl-3-p-tolylsulfonyl urea on the glucose-6-phosphatase activity of a rat liver homogenate. However, Hawkins, *et al.*(17) reported that oral administration of carbutamide to rats for a three-week period decreased the glucose-6-phosphatase activity of a liver homogenate prepared from these rats. No information was given regarding an immediate effect of carbutamide on the glucose-6-phosphatase activity of liver. Since the effect of carbutamide on blood sugar is observed within 2 hours, the lowered level of glucose-6-phosphatase reported by Hawkins may have no direct relationship to the hypoglycemic activity of the drug.

Summary. The concentrations of carbutamide in the liver and plasma of rats receiving a wide range of doses of the drug have been determined. When carbutamide was added to a rat liver microsome preparation in these concentrations no inhibition of glucose-6-phosphatase activity was observed.

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Benzyldimethylbufotenin, a Powerful Antimetabolite of Serotonin. (22712)

E. SHAW AND D. W. WOOLLEY
(With the technical assistance of E. Van Winkle)
Rockefeller Institute for Medical Research, New York City

Although many antimetabolites of serotonin, capable of antagonizing specifically the actions of this hormone, have been discovered (1,2,3,4) only a few have proven to be active in whole animals. Most of them act only in isolated tissues, or exhibit only low potency in intact mammals(2,4). Even among those capable of affecting the whole animal many are only effective when administered parenterally, and fail by the oral route. Yet it is necessary to develop orally active compounds, as has been pointed out earlier(5), if useful drugs related to serotonin are to be achieved. Consequently we would like to report studies of a new antimetabolite of serotonin which is highly active in combatting the pressor action of this hormone, and one which is very effective even when fed.

The new antimetabolite is 1-benzyl-2, 5-dimethylbufotenin hydrochloride, which may be abbreviated to "benzyl analog of bufotenin" or BAB. The structural formula, as well as that of serotonin, is shown in Fig. 1. Here also is shown the benzyl analog of serotonin (BAS) which recently(4,6) has been found to be a highly active antiserotonin, capable of

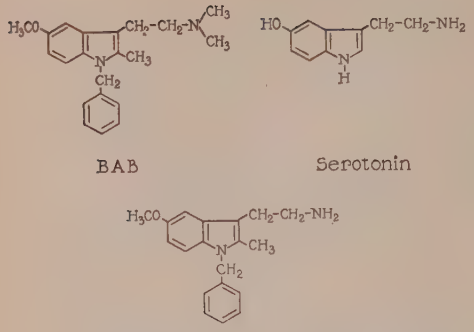


FIG. 1.

protecting dogs by the oral route from the pressor action of serotonin. BAS has also proved to be capable of lowering the blood pressure of humans suffering from hypertension(6,7), and to do so without producing marked side effects. The close relationship of the new antimetabolite to BAS is evident, hence some differences in the biological properties of BAB and BAS are noteworthy.

Preparation of BAB. BAB was synthesized as previously described(8). An unfortunate typographical error in reference(8) caused this compound to appear in the table without its methyl group at position 2. Thus, in Table II on page 4321 of the chemical paper the 12th compound should read 1-benzyl-2-methyl-5-methoxy-N, N-dimethyltryptamine hydrochloride instead of 1-benzyl-5-methoxy-N, N-dimethyltryptamine hydrochloride. A modification was introduced into the scheme of synthesis which facilitated large scale operations. This was to conduct the benzylation at the amide stage rather than at the hydrazine step. BAB in the form of its hydrochloride was soluble in water to about 40 mg per ml. In Ringer's solution or other chloride-containing aqueous solutions, it was soluble only to about 4 mg per ml. All solutions for biological testing were in Ringer's solution.

Protection of dogs intravenously against pressor action of serotonin. The tests were conducted exactly as described previously for other antiserotonins(4). Normal dogs, anesthetized with Nembutal were connected to a mercury manometer via the femoral artery, and were calibrated as to their pressor responses to graded doses of serotonin administered intravenously. Small doses of BAB (10 μg per kg) were injected intravenously into the jugular vein. The animals were then challenged again with a dose of serotonin, which, in their untreated state, had been sufficient to evoke a pressure rise of 20-40 mm of Hg. Changes in blood pressure were recorded, and if there had been no protection against the pressor effect, or if the protection was not complete, the dose of BAB was increased and the challenging repeated. Thus an estimate of the amount of BAB required to protect each dog was made. Data for 3 dogs so tested are shown in Table I. Very small doses indeed (8-12 μg per kg) were capable of causing partial protection. However complete protection was difficult to achieve, although doses of 1 mg per kg approached this goal.

In 2 additional dogs (not shown in the table) which had given abnormal responses to serotonin, the effect of BAB was less easy to assess. For example dog 35 had shown only small pressor responses to serotonin.

TABLE I. Changes in Arterial Pressure of Dogs Given BAB and Serotonin Intravenously.

Dog No.	I.V. serotonin (μg/kg)	I.V. analog (μg/kg)	Interval between analog & serotonin, min.	Initial pressure (mm Hg)	Maximal pressure increase (mm Hg)
7	62	0	—	102	28
	62	12	15	—	14
	62	120	15	—	12
	62	120	65	—	20
	62	1200	60	—	10
29	40	0	—	92	38
	40	8	15	—	24
	40	80	15	—	30
	40	80	75	—	38
	40	800	15	—	18
	40	800	94	—	38
34	80	0	—	116	18*†
	80	8	15	—	12
	80	1200	15	—	6

* All responses in this dog were obtained after intrav. inj. of atropine sulfate 1.2 mg/kg which was administered to obliterate the marked hypotension caused by serotonin alone.

† After each inj. of serotonin there was a very fleeting spike of pressure both before and after BAB. This response was not included in the results shown. Only sustained rises in pressure were considered.

TABLE II. Changes in Blood Pressure in Dogs Challenged with Serotonin before and after They Had Been Fed BAB.

Dog No.	Analog, mg/dog/day	I.V. serotonin challenge, μg/kg	Initial pressure	Maximal fall in pressure	Maximal rise in pressure
			mm Hg		
19	0	37	76	8	36
	10	37	78	0, 8*	26, 4*
	10	74	—	2	14
29	0	40	92	0	38
	10	40	106	20, 12*	2, 14*
32	0	71	96	0	24
	0	142	—	0, 8*	24, 30*
	10	71	70	0, 20*	22, 6†
	10	142	—	20	10

* The 2 numbers represent responses to 2 successive challenges with serotonin.
† The response to the first inj. of serotonin was followed by a rising baseline (Fig. 4, ref. 5). Subsequent challenges gave only slight rises.

These consisted of a tiny sharp spike (16 mm, 15 sec. duration) followed by a small sustained rise lasting several minutes. Larger doses of serotonin (up to 222 μg per kg) did not produce a more typical response. Administration of BAB (1.2 mg per kg) protected this dog against the sustained rise caused by serotonin, but greatly increased the height of the sharp spike (72 mm).

The protecting action of BAB appeared to be shortlived by the intravenous route. Data of Table I show that the protection had largely disappeared after about an hour. This was in contrast to the action of BAS in which case it has been found that full protection developed slowly and required about an hour after intravenous administration to become evident.

Protection of dogs by the oral route. The design of the experiments was the same as that used by Shaw and Woolley(4,5). Dogs which had been calibrated with serotonin were fed BAB once daily for 4 days. They were then challenged with serotonin again, and changes in arterial pressure were recorded and compared to those in the unprotected state. A dose of 10 mg of BAB per dog per day was found adequate to protect the animals from the pressor effects of serotonin. Data from 3 such experiments are shown in Table II.

Toxicity of BAB. The dogs which had eaten, or had been injected intravenously with BAB, showed no discernible toxic effects.

A more extensive study of toxicity was made in mice. For the acute tests, groups of 5 to 12 adult Swiss mice of the Webster strain were injected intraperitoneally with graded doses of BAB. With 100 mg/kg a flaccid paralysis of the hind legs appeared a few minutes after injection, succeeded by convulsive seizures which passed off after about an hour, followed by a period of lethargy, then complete recovery. Repetition of this dose on the following day again precipitated the signs which were again followed by recovery. After 5 days of this treatment, one of the 5 mice was unable to walk 24 hours after the last injection. Autopsy showed swollen and hemorrhagic intestines in this mouse but not in others. With 40 mg per kg only minimal signs were seen, most prominent of which were some difficulty in use of hind legs, some hyperventilation and lethargy or sleepiness which developed about half an hour after the injection. The behavioral changes caused by the large doses (100 mg/kg) could be summarized as excitement of the central nervous system, quickly followed by tranquilization.

Tests of chronic toxicity on groups of 12 adult mice were made by daily injection of 40 mg/kg intraperitoneally. Aside from slight motor difficulties with the hind legs which were noticeable for a short time after each injection, these animals showed no ill effects. They maintained their weights for the duration of the experiment (4 weeks). At this time autopsy revealed no changes which could be recognized on gross examination. The acute and chronic toxicities of BAB were therefore somewhat less than for BAS(4).

Antiserotonin action of BAB on isolated rat

uterus. The antiserotonin action of BAB was tested in the way described for other substances(4) on the isolated uterus of estrogen-treated rats. In this system the compound prevented the contractions induced by serotonin. At 0.1 μg per ml BAB prevented the effects of 0.01 μg of serotonin per ml. Quantitative comparison of the effectiveness of BAB with that of BAS was difficult because the action was irreversible, and the potency depended on the time of exposure of the tissue to the analog before the challenging dose of serotonin was applied. These difficulties also were a prominent feature of the action of BAS, as has already been described in detail (4). The chief conclusion to be drawn from these studies was that BAB was a potent and irreversible antagonist of serotonin on the rat uterus.

Differences in action between BAB and BAS.

(a) *Taste.* BAS when applied to the tongue as solid crystals had little if any taste. BAB, on the other hand, when similarly applied had a most pronounced taste, and caused a numbing sensation which persisted and spread to adjoining areas of the tongue, and which lasted for several hours. It was reminiscent of the similar sensation caused by pyribenzamine. When one considers that BAS is a primary amine which should be attacked by amine oxidase, whereas BAB is a tertiary amine which should not be thus attacked, one may suggest that amine oxidase may play a role in the protection of nerves from the primary amine. Several of the older antimetabolites of serotonin such as the 5-aminoindoles(1) which should not be subject to amine oxidase numbed the tongue just as BAB did. The need for keeping antiserotonin out of the nervous system, for example in order to prevent psychiatric disorders, has been clearly recognized(6). A clue as to how this is to be done may be furnished by the present example which shows that, at least in the tongue, the presence of a primary aliphatic amine is necessary in an analog to keep it away from the neural structures.

(b) *Hypotension on intravenous injection.* Intravenous injection of BAS into dogs(4) or into men(7) did not result in hypotension. By contrast, doses of BAB 10 mg or greater per dog (1 mg per kg) always caused a sharp fall in arterial pressure. This was 10-40 mm of Hg (average of 6 dogs, 24 mm of Hg). The hypotension was short-lived (1-2 minutes).

(c) *Hind leg paralysis.* Mice given more than 40 mg of BAB per kg exhibited the transient paralysis of the hind legs already described. This has not been seen after treatment with BAS. This provided further evidence for an effect of BAB on nerves.

(d) In a preliminary clinical trial of BAB given orally to hypertensive patients, Drs. Robert Wilkins and William Hollander of Boston have not found it to possess the significant hypotensive effect shown by BAS at comparable dosage.

These differences in two compounds so similarly constituted, and so similar in several other biological properties tended to focus attention on the role of the primary amino-group in the designing of antiserotonins for specified pharmacological effects.

Summary. The benzyl analog of bufotenin, BAB, or 1-benzyl-2,5-dimethylbufotenin (1-benzyl-2-methyl-5-methoxy-N, N-dimethyltryptamine hydrochloride) has been found to be a highly active antagonist of serotonin. Not only was it effective on isolated tissues, but, unlike many others, was highly active as an antagonist to the pressor effects of serotonin in dogs, even when given by the oral route. Some differences in action from the corresponding primary amine (benzyl analog of serotonin) were found.

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Total Serum Glycoprotein Levels in Normal New Zealand White Rabbits.*† (22713)

R. G. Graham,‡ J. D. Allen, H. L. Dobson, and W. J. Fahlberg

Departments of Medicine and Microbiology, Baylor University College of Medicine, Houston, Texas

Interest in the serum glycoproteins has been aroused by reports of abnormally high levels in various physiological and pathological states(1,2). Although the levels of these carbohydrate-containing proteins of the serum have been studied in many conditions and disease states, and work on establishing the chemical composition has been initiated, little is known about the physiological significance of these substances and from whence they arise(3,4).

Inasmuch as it will be necessary for laboratory animals to be used in experimental studies designed to establish the site or sites of the origin of these compounds and to determine the physiological and pathological significance of serum glycoproteins, the present investigation was undertaken to establish the normal level of serum glycoproteins in New Zealand white rabbits. In our experience, the rabbit has proven to be the most satisfactory laboratory animal for this type of study because of the similarity between its electrophoretic serum protein patterns and those of the human in both the normal and infected state.

Methods. In this series, 10 male and 9 female New Zealand white rabbits weighing from 2.3 to 2.8 kg each were used. They were individually housed in wire cages in an air conditioned room and were maintained on a diet of Purina rabbit pellets supplemented daily with carrots and tap water, *ad lib.* All animals were observed for a period of 6 weeks prior to this study in order to rule out any abnormalities. Blood samples were collected weekly on each normal healthy animal for a period varying from one to 11 weeks with the different animals. Approximately 5 cc of blood was obtained each week by lancing the ear and allowing free-flow collection in acid cleaned test tubes. With the exception of the first 3 weeks all samples were stored in the frozen state between determinations; in our experience serum specimens showed no demonstrable change for as long as 3 months. The total serum glycoproteins were determined according to a modification of Winzler's technic for protein-bound hexose determination in which 90% sulfuric acid was substituted for the 60% acid in mixing the orcinal reagent(1). Each sample was determined in duplicate on at least 2 separate occasions and the values used in the following results were derived from the average of all determinations on each sample. The mean, standard error of the mean, t, and probability values were determined by the standard statistical procedures.

Results. In 122 serum samples, the established mean and standard error for total serum glycoproteins was 84.5 $\pm$ 1.31 mg% protein-bound hexose ($p<.001$). The normal range was 56.5 to 113.5 mg% protein-bound hexose. Summaries of the results of the chemical analyses for total serum glycoproteins showing variation between individual animals and effects of weekly bleedings are presented in Tables I and II, respectively. All animals continued to gain weight throughout the course of the experiment and there were no apparent after-effects due to bleeding. No statistically significant changes occurred in the concentration of the serum glycoproteins from week to week when compared to the established mean. When compared to the first week's mean, however, there was a significant increase in the total serum glycoprotein at the sixth bleeding. No significant difference was found between sexes.

Discussion. On the whole, the variation between determinations in each animal appeared to be greater than that between the

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‡ Medical Student Fellow, U.S.P.H.S.

TABLE I. Variation between Individual Animals, Grouped According to Sex.

Male No.	No. samples	Mean ± S.E.*	Female No.	No. samples	Mean ± S.E.*
1	10	83.3 ± 3.39	24	8	80.5 ± 6.53
2	10	71.9 ± 2.81	4	10	76.0 ± 2.94
7	10	89.1 ± 5.59	6	10	84.8 ± 3.22
8	11	91.6 ± 4.68	25	9	85.6 ± 4.86
10	11	86.2 ± 2.77	12	11	90.6 ± 4.69
Avg		84.4 ± 3.42	Avg		83.5 ± 2.45

* All serum glycoprotein levels are expressed as mg % protein-bound hexose.

individual animals. The normal range varies considerably, much like blood sugar levels, but in Table I less variation is seen between the individual animals. Little change is noticed in the levels of glycoproteins in the group from week to week when compared with the average mean so that it is assumed there was no significant effect on the blood levels of glycoproteins due to the bleeding. However, when the concentrations of glycoproteins were compared to the mean of the first week a significant P value was obtained for the sixth week. We are unable to explain this observation on the basis of dehydration as total protein determinations were not done. Since the rabbits were housed in air conditioned quarters and always had ample water supply, it was not felt that dehydration was a contributing factor. On the basis of work by Weimer *et al.*(5) it appears doubtful that significant variations in total protein occur normally. They showed no significant increases, and only transient decreases in the serum proteins of guinea pigs and rats following weekly bleedings of approximately 20% of the total blood volume. Variation between animals was greatest at the third bleeding but this is unexplained and cannot be attributed to environmental changes, in that the date of the third bleeding was not the same among all rabbits. There was no significant statistical difference between the male and female groups ($P = < .001$).

TABLE II. Effects of Weekly Bleedings on Serum Glycoprotein Concentrations.

Wk No.	No. samples	Mean ± S.E.*	P†
1	9	82.9 ± 3.59	—
2	10	86.0 ± 3.06	> .2
3	10	83.7 ± 9.14	> .5
4	10	81.9 ± 4.77	> .5
5	9	85.3 ± 3.44	> .3
6	10	89.3 ± 5.00	< .05
7	8	88.6 ± 5.27	> .05
8	10	82.8 ± 3.17	> .5
9	9	82.4 ± 3.83	> .5
10	8	82.9 ± 2.03	>0
11	7	80.1 ± 3.05	> .2

* All serum glycoprotein levels are expressed as mg % protein-bound hexose.

† P values derived by comparing means of each succeeding week with mean of first week.

Summary. The normal serum levels of glycoproteins in 19 New Zealand white rabbits are reported. It was shown that there was no significant difference between the sexes and essentially little effect on total serum glycoprotein concentration when the rabbits were bled weekly in the quantity reported over an 11 week period.

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Axenic Cultivation of *Caenorhabditis briggsae* (Nematoda: Rhabditidae). V. Maturation on Synthetic Media.* (22714)

ELLSWORTH C. DOUGHERTY AND EDER LINDSAY HANSEN

Kaiser Foundation Hospital, Oakland, and Department of Physiology, School of Medicine, University of California, Berkeley

The free-living, soil-dwelling, hermaphroditic rhabditid nematode, *C. briggsae*, can mature under axenic conditions on a variety of media containing ingredients of complex, undefined composition—for example, certain liver fractions(1-3), plasma protein fractions (4), chick embryo juice(1), and extracts of equine pancreas, thymus, or spleen(5). Some of these media have also contained a large number of added known substances(5,6), but, until recently, all have included at high levels certain of the complex, crude substances mentioned. However, we have recently recorded (7) success in rearing *C. briggsae* from larva to larva on a defined medium containing only a trace of the standard liver medium used by us in maintenance of stock axenic cultures of this organism. Now we are able to describe limited success following elimination of even this trace, such that *C. briggsae* can be reported as passing one generation on certain media of synthetic composition.

Materials and methods. Experiments were conducted as follows: 1) young adult worms were selected from stock axenic cultures maintained in standard liver medium, washed through 4 changes of 1/15 M K phosphate buffer (pH 7) in small watch glasses, and left to lay eggs for approximately 24 hours; 2) larvae hatched by the end of this period were taken up singly or in groups of 2 or 3 by micropipette and inoculated into tubes, 10 mm wide by 75 mm long, each containing 0.2-0.3 ml of liquid medium to be tested—generally only one larva was placed in a tube, 3 tubes of each medium being used; 3) tubes were plugged with cotton, sealed with 2 layers of parafilm, and incubated upright in the dark at 20°C in a constant-temperature incubator; 4) each tube was inspected daily, or every 2 to 3 days during less critical periods, placed almost horizontally on a transparent plastic square (with plugged end of tube elevated slightly) and studied under the dissecting microscope at 19.5 x magnification; 5) with each observation a record was kept, of: a) approximate size of each worm during the first generation; b) time at which the first F_1 larva or larvae were seen; c) size range of F_1 larvae during the second generation; d) time of appearance of F_2 larvae; and e) nature of the ultimate population after several weeks of incubation, determined by total or aliquot counts. We succeeded in rearing worms through one generation on 3 synthetic media, 2 being simplifications of the third. The components of the most complex medium (GM-8) are listed in Table I; its compounding is explained in Table II. The reason for using a considerable number of stock solutions in preparing GM-8 was to permit ready preparation of such simplifications of the latter as are illustrated by GM-9 and -11. Their composition is indicated in footnotes to Table I. Recent experiments leading up to, and including, those in which GM-8, -9, and -11 were tested also included testing of other defined media, the details of which will be reported later. We also tested them supplemented with various levels of standard liver medium, consisting of equal parts of liver pro-

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TABLE I. Synthetic Medium* for *Caenorhabditis briggsae*.

Components	mM	mg/l
Amino acids:		
‡L-alanine	12	1070
L-arginine (free base)	4	697
‡L-aspartic acid	10	1331
†‡L-cysteine (free base)	3	363
‡L-glutamate (monosodium)	15	2537
‡Glycine	5	375
L-histidine	4	621
L-isoleucine	8	1049
L-leucine	13	1705
L-lysinium acetate	10	2062
L-methionine	3	448
L-phenylalanine	10	1652
‡L-proline	17	1957
‡L-serine	11	1156
L-threonine	7	834
L-tryptophane	2	408
‡L-tyrosine	1	81
L-valine	11	1289
(Total)	(146)	
Other "structural" compounds:		
Choline diacid citrate	2	590
i-inositol · $2H_2O$	2	432
Nucleic acid substituents		
Adenylic acid	1	347
Guanylic "	1	363
Cytidylic "	1	323
Uridylic "	1	324
Thymine	1	126
Energy source:		
D-glucose	73	13,150

Components	mM	mg/l
Vitamins and growth factors:		
N-acetylglucosamine		2.0
D-alanine		1.0
p-aminobenzoic acid		1.0
Biotin		.5
Cyanocobalamine		.5
Folinate (calcium)		.5
Niacin		1.0
Niacinamide		1.0
Pantetheine		.5
Pantothenate (calcium)		1.0
Pteroylglutamic acid		1.0
Pyridoxal		.5
Pyridoxamine		.5
Pyridoxine		1.0
Riboflavine-5-phosphate (sodium)		1.0
Thiamine chloride · hydrochloride		1.0
§DL-6-thioctate (sodium)		.5
‡Ascorbic acid	1	88
‖*Salts:*		
KH_2PO_4	6	817
KOH	11	617
$Mg \cdot H \cdot citrate \cdot 5H_2O$	2	610
$CaCl_2 \cdot 2H_2O$	1	147
$K_3 \cdot citrate$	1	324
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	.1	39.2
$MnCl_2 \cdot 4H_2O$	.075	14.8
$ZnCl_2$	.05	6.8
$CuCl_2 \cdot 2H_2O$	.025	4.3
†‡H_3BO_3	.01	.62
†‡$(NH_4)_2VO_3$	.01	1.16
†‡$(NH_4)_2MoO_4 + H_2MoO_4$	.01	1.77
†‡KI	.01	1.66
Solvent (for certain vitamins):		
Triethanolamine	.057	8.5

* GM-8. † Omitted for GM-9. ‡ Omitted for GM-11. $Na \cdot citrate \cdot 2H_2O$ at 5 mM (1471 mg per l) and citric acid at 3 mM (576 mg per l) were added to replace Na^+ and acid. § Replaced in GM-9 and -11 by thioctic acid dissolved initially at 20,000× in 5% triethanolamine (TEA).

Expressed as ions:	mM		mM
K^+	20	NH_4^+	.24
[1]Na^+	15	$SO_4^=$	.2
[2]$PO_4^=$	10	Fe^{++}	.1
Mg^{++}	2	Mn^{++}	.075
Cl^-	2	Zn^{++}	.05
Ca^{++}	1	Cu^{++}	.025
		[3]Co^{++}	.00037
$MoO_4^=$, $BO_3^=$, I^-, $VO_3^=$ (each)			.01

[1] As monosodium glutamate.
[2] As nucleotides + KH_2PO_4.
[3] As cyanocobalamine.

tein fraction (LPF-C)†, dissolved in 1/15M K · phosphate buffer, and an autoclaved liver infusion or extract (ALE)‡(4) prepared with the same buffer. It was added to the synthetic media at levels from 50% down to 0.032% of the level used for routine stocks, Finally we tested GM-8 supplemented separately with LPF-C and ALE at 50% and at 10% respectively. Tubes containing 100% liver medium without added ingredients were used as controls.

Results. GM-8, -9, and -11 by themselves permitted slow maturation of certain

† Dry wt 31.88 mg/ml; N content (by micro-Kjeldahl) 2.488 mg/ml. LPF-C prepared in Aug., 1954, and stored in frozen state.

‡ Dry wt, 49.00 mg/ml; N content, 2.519 mg/ml. ALE prepared on 5-23-55 and stored in frozen state.

TABLE II. Stock Solutions* for Medium GM-8.

Stock solutions	Concentrations (relative to full-strength basal medium)
1. "Essential" amino acids	10×
2. "Non-essential" amino acids†	5×
3. D-glucose	20×
4. KH_2PO_4	100×
5. Nucleic acid bases (with KOH)	20×
6. Choline	100×
7. *i*-Inositol	100×
8. Ascorbic acid	100×
9. Vitamins: Group I	1000×
10. " II	1000×
11. " III	1000×
12. Salts‡: Group I	50×
13. " II	1000×

* All made up with triply distilled H_2O, which was also used to bring vol of medium to double strength. Latter then sterilized, usually in 10 ml amounts, by filtration through fritted glass. Stock solutions not obvious from Table I are as follows: 1. 10 amino acids essential to rat. 10. Pyridoxal·HCl, pyridoxine·2HCl, calcium folinate, d-pantetheine. 11. Vitamins requiring initial dissolving in TEA for 20,000 × concentrated sub-stocks: pABA (5% TEA), PGA (5% TEA), niacin (1% TEA), biotin (1% TEA). 13. Salts of B, V, Mo, and I.

† Cysteine omitted; added as dry powder to final solution.

‡ $CaCl_2 \cdot 2H_2O$ omitted; added as dry powder to final solution. KOH omitted; used with nucleic acid bases.

inoculated larvae, with production of eggs by the resulting adults and hatching of F_1 larvae. On GM-11 limited growth of F_1 larvae beyond their size when newly hatched also occurred. With addition to these media of liver medium at 0.1%, maturation of the first generation (P) worms was somewhat faster, and some of the resulting F_1 larvae reached maturity and produced F_2 offspring. With liver medium at 50% the P and F_1 generations were passed rapidly, faster than in controls, but more slowly than under previously established conditions(5) in which *C. briggsae* was grown on nutrient agar slants in monoxenic association with *Escherichia coli*.

These trials, in which GM-8, -9, and -11 were tested with supplements of liver medium at 50% and 0.1% of the standard level, have been the only such experiments so far with 2 of the media—namely, GM-9 and -11. GM-8, however, was further tested with supplements at levels of 10%, 3.2%, 1%, 0.32%, and 0.032%. All worms (some 23) that were followed sufficiently long matured and produced F_1 larvae in GM-8, supplemented with liver medium at all levels down to 0.1%; but at 0.032% this was true for only one of 3 worms. At all levels except 0.032% some of the larvae of almost all progeny groups matured in turn and produced F_2 larvae. Generation I time tended to be progressively longer at progressively lower levels of supplementation with liver medium; and this was more strikingly true for Generation II time.

By contrast with liver medium, its components (LPF-C and ALE), when tested separately, proved far less active as supplements to GM-8. Starting with 3 P larva in each test, we obtained F_1 larvae with levels of both 50% [*i.e.*, the level in liver medium] and 10% for LPF-C and ALE respectively. The 50% level of LPF-C also supported the production of an F_2, but not the 10% level. By contrast, in the case of ALE the reverse was true, although only one out of the 3 tubes at 10% produced F_2 larvae and only in small numbers.

Additional quantitative data are summarized in Table III; and data from the experiments with GM-8 have been used to construct a graph expressing the activity of mixtures of it and successively smaller amounts of liver medium (Fig. 1). The lines shown were calculated by the method of least squares.

TABLE III. Typical Maturation Times in Different Media at 20°C.

Media	Maturation times in days Generation I	Generation II
Synthetic media		
GM- 8	24	—
GM- 9	25	—
GM-11	17	—
Liver medium (LM)	7	5
GM-8 + .1% LM	9	19
" + 1.0% "	9	12
" + 50 % "	4.5	4
" + 50% LPF-C	6	5
" + 10% "	12.5	—
" + 50% ALE	16	—
" + 10% "	25	27
Associated with *E. coli* on nutrient agar	3.5	Not determined

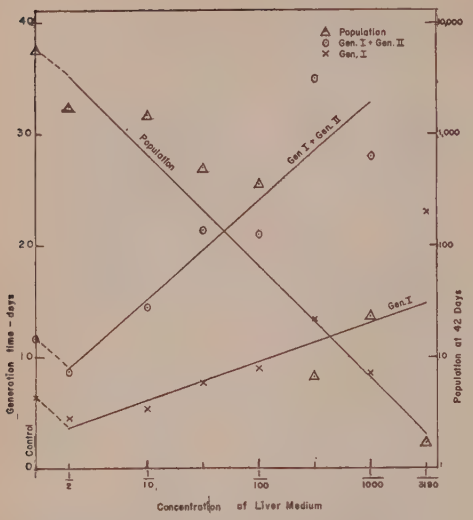


FIG. 1. Maturation time and population level as functions of concentration of liver medium in synthetic medium, GM-8.

Discussion. The foregoing results establish 3 important points: 1) it is possible to obtain slow maturation and reproduction of *C. briggsae* in certain synthetic media of known composition; 2) very small amounts of liver medium (0.032% being the lowest tested) are stimulatory to these synthetic media; but 3) liver medium must be present at much higher levels (approaching 50%) before the growth rate is equal to, or greater than, that in 100% liver medium alone.§

Even though GM-8, -9, and -11, when unsupplemented, are not adequate for indefinite cultivation of *C. briggsae,* the fact that they permit maturation and reproduction is a highly significant advance toward an adequate defined, synthetic medium. Numerous earlier attempts(5,6) with other defined media, including certain media used in tissue culture work (*e.g.,* medium 858 of Healy *et al.* (8)) had met with uniform failure; the P larvae grew very little if at all. It was not until media (GM 1-7, of which the ingredients are not given here) approaching GM-8 in composition were tried that significant growth was observed. Relatively little can be said at present as to the essentiality *vs.* non-essentiality of the various ingredients of GM-8, -9, and -11. An important point in this connection is, however, that GM-11, from which the 8 amino acids that are not required by the rat were omitted, supported better growth than GM-8, which had them.

The results with GM media, unsupplemented and supplemented with liver medium, admit of 3 interpretations: 1) the liver medium supplies fundamental growth factors that the organism cannot make, at least in adequate amounts; 2) it provides substances that have no basic metabolic role, but are needed to reverse certain toxic properties of the synthetic media; or 3) it acts in both ways.

Whatever the case may be, we feel it highly probable that a larva as originally inoculated contains a store of some highly potent substance or substances, which permit maturation of the P generation, but are insufficient to allow progress much beyond that point without the presence of liver medium. Moreover, the fact that traces of liver medium can support a relatively rapid Generation I time, yet provide for but slow growth of the F_1 larvae with a consequently much prolonged Generation II time, suggests that the original worm can efficiently utilize a trace of liver medium as a source of one or more needed substances, absent or inadequately present in the synthetic media, so long as it has an adequate store of the same or other substances carried over from the egg, but that, with the depletion of the latter store, it has difficulty in deriving its needs from the synthetic media supplemented with but traces of liver medium. Finally, the fact that the liver medium must be present at high levels to permit continued growth at a rapid rate beyond the appearance of F_1 larvae may be interpreted to mean that only high levels of liver medium can counteract the effect of depleting the original larva's store of an essential substance, or substances. It is impossible to say at the present time whether the liver medium is itself acting to supply one or more substances

§ We have found the standard liver medium to permit indefinite cultivation of *C. briggsae,* one stock of this organism now being in its fourteenth transfer after 20 months of axenic growth (July, 1956).

that the worm needs in trace amounts and that the liver medium can supply only when present at high levels, or whether some major substituent of the liver medium is needed at high concentrations, or whether both conditions obtain. In other words, it cannot as yet be said whether the liver medium is supplying one or more micronutrients, macronutrients, or both.

The results of separately supplementing the synthetic media with LPF-C and ALE provide important information. Since neither component by itself is nearly so potent as the standard liver medium, which combines the two in equal volumes, there would appear to be 2 requirements, or sets of requirements, involved—both present in each fraction, but one at relatively high levels in LPF-C and the other in ALE. The relative potency of these 2 requirements, or sets of requirements, has not yet been ascertained.

As regards *factor Rb,* a nutritional requirement for *C. briggsae* defined as protein-like and relatively heat-labile(1,7), it appears to be present in both LPF-C and ALE.

C. briggsae is the first nematode to be grown on a completely defined medium through an entire generation. Certain nematodes not belonging to the family Rhabditidae are known to be capable of axenic growth on complex media similar to our liver medium—for example, *Neoaplectana glaseri*(9); the vinegar eelworm, *Turbatrix aceti*(10) and the free-living larval stages of the dog hookworm, *Ancylostoma caninum*(11). Recently Weinstein(12) has succeeded in rearing the trichostrongyle, *Nippostrongylus muris,* of the rat to maturity on an even more complex though cell-free medium. All these species belong to the nematode order Rhabditida. It would be of great interest to test them for their capacity to grow on media similar to GM-8, with the ultimate goal (among others) of devising a rational nematocide. We ourselves have tried to grow a plant pathogen, *Ditylenchus dipsaci* (order Tylenchida), on a medium similar to GM-8, supplemented with liver medium at the 10% level, but we observed no growth of the larvae introduced into this medium.

Summary. 1. Newly hatched larvae of *Caenorhabditis briggsae* can grow to adulthood and F_1 larvae can be produced on a completely defined synthetic medium (GM-8) consisting of 18 L-amino acids, D-glucose, 4 ribonucleotides plus thymine, choline, *i*-inositol, ascorbic acid, 17 "trace" vitamins and growth factors, and a mixture of salts. 2. Media (GM-9 and -11) like GM-8 except for certain omissions (and minor additions to restore acid-base balance and salt levels) also support maturation and reproduction. 3. In the case of GM-11, omitted substances include the 8 "non-essential" amino acids. 4. The presence of a trace amount of the standard liver medium used for the indefinite axenic cultivation of *C. briggsae* is markedly stimulatory to the synthetic media, and some F_1 larvae mature and produce F_2 larvae when liver medium is present at only 0.032% of its standard strength. 5. For sustained growth, however, *C. briggsae* requires the presence of much higher levels of liver medium—between 10 and 50% of the standard level used for stock cultures. 6. Evidence is here presented that the liver medium contains 2 requirements, or sets of requirements, not present in the synthetic media, at least in adequate amounts or proportions.

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Fate and Distribution of Fe^{59} Labelled Ferrocyanide in Humans and Dogs.* (22715)

CHARLES R. KLEEMAN AND FRANKLIN H. EPSTEIN (Introduced by P. K. Bondy)

Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

Previous studies(1,2,3) have demonstrated that unlabelled ferrocyanide ion fulfills many of the criteria required of a substance which would measure the volume of the extracellular fluid. After intravenous injection it equilibrates rapidly in a "space" approximating 20% of body weight; it does not penetrate or cannot be detected in red blood cells, gastric juice or feces; and from 94 to 98% of administered dose can be recovered in urine in 24 hours. In addition, Berliner and his associates(4) were unable to demonstrate by dialysis any binding of ferrocyanide to plasma proteins.

Unfortunately the quantities of non-radioactive ferrocyanide which must be used in "space" measurements were found by Miller and Winkler(5) to be nephrotoxic in humans. The present investigation was, therefore, undertaken to evaluate the fate and distribution in humans and dogs of small quantities of sodium ferrocyanide labelled with Fe^{59}.

Materials and methods. Studies *in vivo* were carried out in 9 human subjects and 3 nephrectomized dogs. The former included 4 male medical students, 2 patients with ascites and anasarca, one cirrhotic without ascites or edema and 2 patients with severe renal failure secondary to chronic pyelonephritis. In all experiments 30-50 mg of Fe^{59}-labelled sodium ferrocyanide (approximately 75 μc) were injected intravenously from a calibrated syringe. Measured amounts of inulin were also injected into the 3 dogs and in one subject with renal failure. Arterial samples of blood from the dogs and venous samples from the humans were collected at intervals varying from 10 minutes to one hour. Time required to reach equilibrium and the rate of disappearance of radioactivity from the blood were determined and the apparent volumes of distribution at equilibration were calculated by extrapolating back to zero time in the usual way†(1). An aliquot of the injected solution was counted simultaneously with all biologic samples, to correct for physical decay. In most studies urines were collected for 24 to 48 hours to determine the recovered radioactivity, and in addition, in 5 experiments hourly urine collections were made to determine simultaneous renal clearances of creatinine and radioactive ferrocyanide. Radioactivity in the 48-hour stool collections of 3 subjects and in the gastric juice and saliva of one subject was also measured. The distribution of radioactive ferrocyanide between plasma and red blood cells was determined *in vivo* and *in vitro* utilizing technics previously described(1). The diffusibility of radioactive and non-radioactive ferrocyanide from aqueous solutions and plasma *in vitro* was evaluated by two technics: 1) a simple dialysis procedure in which the sample to be dialyzed was placed in a bag of Visking membrane and dialyzed against a solution of isotonic saline for 24 hours with continual shaking, and 2) the anaerobic ultrafiltration technic of Lavietes(6). The *in vivo* and *in vitro* binding of radioactive ferrocyanide by specific plasma proteins was determined by electrophoresis of samples of serum on paper at pH 8.6 and 7.4 in acetate buffer (.04M) on a Spinco apparatus at 0.6-0.7 MV. Duplicate strips of each sample of serum were run simultaneously. One was stained with bromophenol blue dye to locate the distribution of

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† The Donnan factor for Fe $(CN)_6^{=}$ is 1.22, *i.e.* the concentration of Fe $(CN)_6^{=}$ in interstitial fluid is 1.22 × (concentration in plasma water). Since approximately 25% of extracellular water is contained in plasma at equilibrium, the "Average" concentration of Fe $(CN)_6^{=}$ in extracellular fluid (plasma water + interstitial water) equals $\frac{(3 \times 1.22) + (1 \times 1.00)}{4} = 1.16 \times$ (concentration in plasma water).

the proteins, the other was cut transversely into 2 cm. pieces, and both were counted in a well-type scintillation counter. A solution of $Fe^{59}(CN)_6^{\equiv}$ in isotonic saline was simultaneously electophoresed to determine the distribution on the paper of non-protein-bound ferrocyanide ion. Total proteins were precipitated from sera using 10% trichloracetic acid. All samples were counted in duplicate for one to 5 minutes depending on the level of radioactivity. The minimal counts exceeded those of the background by at least four times. The average background with the apparatus used was approximately 115 counts per minute. Plasma and transudate proteins were determined by the biuret technic(7); inulin, endogenous creatinine, and non-radioactive ferrocyanide by methods previously described(1).

Results. Urinary recovery and renal clearance of radioactive ferrocyanide: In the 4 normal subjects an average of 80% (68-87%) of administered radioactivity was recovered in 24 to 48 hours. This contrasted sharply with the 94 to 100% of non-radioactive Fe $(CN)_6^{\equiv}$ recovered in 24 hours when administered to normal dogs(1) and infants (3). In Table I are listed the simultaneous renal clearances of creatinine and $Fe^{59}(CN)_6^{\equiv}$ in 4 normal subjects and one patient (M.S.) with severe chronic renal disease. The ferrocyanide clearance ranged from 20 to 53% of the concomitant creatinine clearance. The highest percentage was in the subject with renal disease.

Distribution between plasma and red cells.

TABLE I.* Simultaneous Clearances of Creatinine and $Fe^{59}(CN)_6^{\equiv}$.

Subject	Creatinine clearance, cc/min.	Ferrocyanide clearance, cc/min.
C.K.	118	41
R.S.	130	48
M.W.	122	34
M.G.	125	25
M.S.	5.6	3.0

* Each value represents the mean of 4-5 consecutive periods of urine collection. Creatinine and Fe $(CN)_6^{\equiv}$ clearances were corrected for serum water; Fe $(CN)_6^{\equiv}$ clearance has also been corrected for a Donnan factor of 1.22.

TABLE II. Difference between Measured and Expected Concentrations* of $Fe^{59}(CN)_6^{\equiv}$ in Whole Blood.

Samples	% difference (expected-measured)
7 human *in vivo*	$+3.6 \pm 3.9$†
3 " *in vitro*	$+ .3 \pm .7$
4 dogs *in vivo*	$+1.0 \pm 2.4$

* "Expected concentration" in cpm/cc whole blood = (plasma concentration) (1-Het), assuming no penetration of red blood cells by radioactive ferrocyanide in the plasma.
† Stand. dev.

Absence in gastrointestinal secretions. Table II: As had been previously noted with non-radioactive ferrocyanide(1), there was no significant penetration of the radioactive ion into the red cell. This held true whether the tracer was added *in vitro* to whole blood at 4° and 37°, or injected intravenously into humans and dogs. In addition, when red cells, from whole blood which had been labelled with ferrocyanide, were washed with cold isotonic saline and then resuspended in saline they contained no appreciable radioactivity. No significant radioactivity was detected in pooled feces, saliva, or gastric juice of humans or dogs.

Disappearance curves and apparent volume

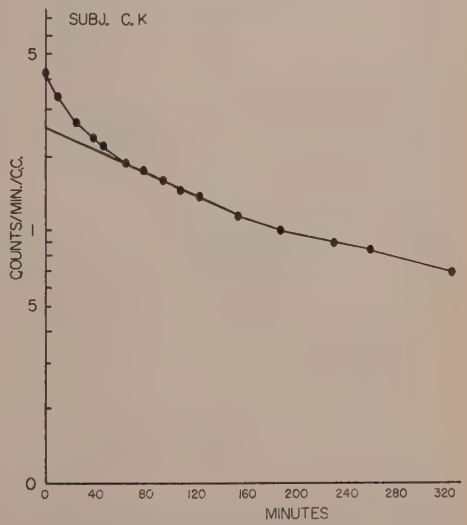


FIG. 1. Semilogarithmic disappearance of Fe^{59} labelled ferrocyanide from the plasma of a normal subject.

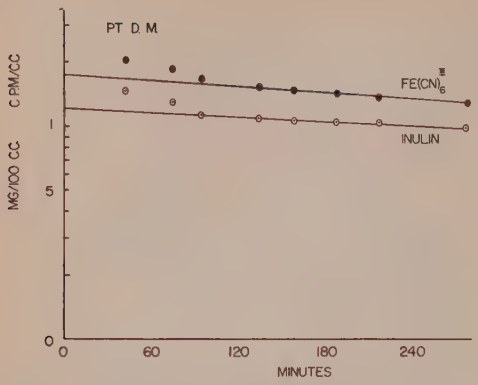


FIG. 2. Simultaneous semilogarithmic disappearance of Fe^{59} labelled ferrocyanide and inulin from the plasma of a patient with severe chronic renal disease.

of distribution in humans and nephrectomized dogs. Fig. 1 illustrates the typical semilogarithmic curve of disappearance seen in a normal subject after apparent equilibration. The disappearance curve was linear between 60 and 160 minutes. Subsequent points fell slightly above the extrapolation of the 60-160 minute line. This lack of linearity after 160 minutes was similar to that seen in normal dogs after injection of non-radioactive ferrocyanide(1). As would be expected; the rate of disappearance of the tracer was much slower in a subject with severe chronic renal disease (Fig. 2). In contrast to normal subjects, neither patient with renal disease demonstrated a changing slope in the late portion of the curve. The similarity between the rate of disappearance of inulin and ferrocyanide is apparent (Fig. 2).

TABLE III. Apparent Volumes of Distribution of Ferrocyanide and Inulin.

Subject	$Fe^{59}(CN)_6^{\equiv}$, % body wt	Inulin, % body wt
C.K.	14.0	
M.G.	15.0	
M.W.	12.7	
R.S.	10.4	
M.S.*	17.0	
D.M.*	20.0	22.5
Dogs		
1	17.1	15.1
2	16.4	19.8
3	10.6	14.0

* Subjects with chronic renal disease.

In Table III are listed the apparent volumes of distribution of radioactive ferrocyanide as well as the volume of distribution of inulin in the cases where this was determined. Only in the two subjects with chronic renal disease did the ferrocyanide "space" exceed 15% of the body weight. Both of these patients were undernourished and had slight ankle edema. Two of the normal subjects (M.W. and R.S.) had ferrocyanide "spaces" which were obviously abnormally small. In 2 of the dog experiments and in one of the patients with renal failure the inulin space actually exceeded the simultaneously measured radioactive ferrocyanide "space." These findings are in marked contrast to those of studies utilizing large (1-1.5 g) doses of non-radioactive ferrocyanide in which the ferrocyanide space always exceeded the inulin space and averaged 20% of body weight(1,2). One explanation of this discrepancy might be partial binding of the tracer dose of ferrocyanide to plasma proteins, resulting in a higher apparent concentration of ferrocyanide in plasma water than in other extracellular fluids.

To investigate this possibility *in vivo*, $Fe^{59}(CN)_6^{\equiv}$ was injected intravenously into 2 subjects with anasarca. Samples of plasma, ascitic, pleural and peripheral edema fluid were simultaneously removed at intervals of two to 36 hours after injection. The results have been expressed in Table IV as the ratio of cpm/cc of transudate water to cpm/cc of plasma water.

At equilibrium, correcting for the Donnan factor, the ratio should approximate 1.22. At no time did the ratio exceed 1.00. This held true even when the plasma concentration had fallen below a level previously attained in the transudate. This strongly suggested *in vivo* binding of radioactive ferrocyanide to plasma proteins.

Direct measurement of binding to plasma proteins: Dialysis and ultrafiltration technics were found unsatisfactory for evaluating the problem of protein binding. When samples of plasma containing radioactive ferrocyanide were dialyzed against isotonic saline for as long as 48 hours very little radioactiv-

ity could be detected in the saline. Similarly, when the plasma was ultrafiltered under a positive pressure of 35 cm of mercury, less than 30% of the expected radioactivity crossed the membrane. These observations did not necessarily indicate binding of ferrocyanide to protein. Surprisingly enough, aqueous solutions of radioactive ferrocyanide similarly did not freely diffuse or filter across a Visking membrane. This could not be corrected by dissolving the tracer in isotonic saline rather than water or by soaking the Visking membrane for 24 hours prior to use in non-radioactive ferrocyanide. Large amounts of residual radioactivity could be detected on the membrane after the latter had been washed for 2 to 3 minutes in running water.

As a result of these findings, electrophoretic and protein-precipitation technics were utilized. When aqueous solutions (water or saline) of radioactive ferrocyanide were electrophoresed at pH 8.6 and 7.4 no significant quantity of radioactivity was detected on the paper with the exception of the end dipping into the anodal bath. In contrast, when serum containing radioactive ferrocyanide was electrophoresed at pH 8.6 approximately 5% of the applied radioactivity was found to migrate with the albumin fraction, and at pH 7.4, approximately 12-18% of the activity migrated with this fraction. No appreciable binding to the globulin fractions was noted.

Little attempt was made in the present investigation to study quantitatively the nature of this binding. However, when Fe^{59} $(CN)_6^{\equiv}$ was added to serum samples containing increasing amounts of non-radioactive ferrocyanide the fraction bound to albumin remained approximately 5% (pH 8.6) at final ferrocyanide concentrations of 1, 2, 5 and 10 mg per 100 cc. At 50 mg per 100 cc the fraction bound fell to 1%. These findings suggest that increasing absolute amounts of ferrocyanide ion are bound up to about 10 mg per 100 cc and that above this level probably little additional binding occurs.

Further evidence of protein binding was obtained by precipitating the plasma proteins with 10% trichloracetic acid. Approximately 25% of the total radioactivity in 1 cc sample of serum remained bound to the precipitated proteins after the latter had been washed 3 times in trichloracetic acid.

Discussion. In the present study the assumption was made that the radioactivity recovered in serum, urine and transudates represented the injected Fe^{59} as ferrocyanide and that the latter was not appreciably altered in the body. The quantitative recovery of non-radioactive ferrocyanide in previous studies (1,3) suggest that this assumption is a reasonable one.

The rate of disappearance of the labelled ferrocyanide from its initial volume of distribution can be directly determined from Fig. 1. In this normal human subject the half-time value (T ½) was approximately 135 minutes. In contrast, the T½ for unlabelled ferrocyanide ion in normal dogs is approximately 40-50 minutes(1). In spite of the slower rate of disappearance in the human experiments linearity was not maintained after 160 minutes. This observation would itself constitute a strong objection to the use of the single injection technic as a method of measuring extracellular fluid volume with this tracer. One explanation for this phenomenon is that the very rapid fall in the level of ferrocyanide in the plasma causes a changing disequilibrium between the plasma and the other extracellular compartments(1). At higher concentrations, rapid renal excretion results in an increasing gradient between these compartments while at low levels the rate of transfer of Fe $(CN)_6^{\equiv}$ from interstitial fluid to plasma may exceed the rate of excretion by the kidney. When renal excretion of ferrocyanide is impaired, as in subjects with renal disease (Fig. 2), the conditions of equilibrium are approached more closely, and a slow, linear disappearance rate is maintained.

The most important reason for the more rapid rate of disappeaarnce observed in dogs as compared to humans(1) is the higher renal clearance of ferrocyanide ion in this species. Numerous studies in the dog(4,8) have demonstrated that the clearance of ferrocyanide is equal to the glomerular filtration

TABLE IV. Ratio of Concentration of $Fe^{59}(CN)_6^{\equiv}$ in Transudate Water/Concentration in Plasma Water.

Subject	Fluid	Time (hr)	Ratio*
M.P.	Ascitic	2	.22
		8	.63
		24	.90
		36	.90
P.G.	Edema	2	.42
		24	.85
		36	.90
P.G.	Pleural	2	.26
		24	.65
		36	.87

* Expected ratio at equilibration = 1.22.

rate. The results of the present investigation confirm the observations of Miller and Winkler(5) that the renal clearance of ferrocyanide in humans is considerably lower than the concomitant clearance of endogenous creatinine. The absolute values listed in Table I must be accepted with reservation because of the probability of significant binding of the radioactive ferrocyanide ion by the plasma proteins.

In spite of the fact that radioactive ferrocyanide did not penetrate red blood cells or enter gastrointestinal secretions, the demonstration of appreciable *in vitro* binding to plasma albumin makes it an unsuitable tracer substance for the measurement of extracellular fluid. The abnormally low volumes of distribution (Table III) and the failure of equilibration with either pleural, ascitic or edema fluid (Table IV) would support this contention. The reported similarity between the initial volume of distribution of ferrocyanide and inulin "space" or "extracellular fluid space" when large amounts of unlabelled ferrocyanide are injected is probably explained by saturation of the ferrocyanide binding capacity of plasma protein at relatively low plasma concentrations (10 mg %) of ferrocyanide. When the absolute amount of ferrocyanide injected approaches tracer quantities, however, the fraction bound to protein becomes appreciable and introduces a significant error.

The demonstration by electrophoresis that at pH 7.4, 12-18% of the added radioactivity migrated with the albumin fraction must be considered at best a rough approximation of the magnitude of the binding which might have occurred *in vivo*. The inability to use dialysis or ultrafiltration technics and the relatively low level of radioactivity in the blood at equilibration prevented adequate study of protein binding in the *in vivo* experiments.

The character of the protein-ferrocyanide bond is not clear. It does not represent combination of iron with the iron-binding-protein of the plasma, for the latter is a β globulin (9). It may represent an example of the tendency of plasma proteins to bind a large number of inorganic anions. Human serum albumin has been shown to combine according to the law of mass action with several inorganic anions(9). This binding is to the positive charges in the protein molecule and decreases as the pH of the solution increases. A similar effect of pH was evident in the present study, since less ferrocyanide was bound at pH 8.6 (5%) than at pH 7.4 (12-18%).

Summary. 1) The fate and distribution of Fe^{59} tagged ferrocyanide were evaluated in humans and dogs. 2) The tracer could not be detected in red blood cells, gastric juice, saliva or feces. 3) The apparent volume of distribution of the tagged ferrocyanide was frequently smaller than the assumed extracellular space, and in 3 instances was less than the simultaneously measured inulin space. 4) *In vitro* binding to plasma albumin was demonstrated by electrophoretic and protein precipitation technics. 5) Because of its binding to plasma protein and rapid excretion by the kidneys, radioactive ferrocyanide is considered unsuitable as a measure of extracellular fluid.

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Modification of Viral Synthesis in Tissue Culture by Substituting Pyruvate For Glucose in the Medium.* (22716)

ALVIN S. LEVINE, PHYLLIS H. BOND, AND HARRIET C. ROUSE
(Introduced by M. D. Eaton)
Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

A previous publication(1) has shown that maximal propagation of the PR-8 strain of influenza virus by *in vitro* cultures of chorioallantoic membrane was dependent on readily available sources of energy. Recent studies revealed that penetration of the virus and utilization of cellular energy for viral synthesis were dependent also on adequate concentration of K^+, and that pyruvate provided a more sensitive system than glucose for studying relation of potassium deficiency to virus synthesis(2). It has now been observed that, while glucose as sole carbon source for cells permits them to propagate both the parent WS and the derived neurotropic NWS strains of influenza virus, the substitution of pyruvate for glucose as carbon source permits propagation of only the parent strain WS. It appears the differences between these two strains depends not solely on host cells chosen, but also upon subtle differences in relation between viral synthesis and sources of energy for the cells.

Materials and methods. Virus. Influenza A viruses, strains WS(3) and NWS(4), were maintained by propagation in the allantoic sac of 10- to 11-day-old chicken embryos. All viral inocula were from allantoic fluid stored in hermetically sealed vials in a dry ice chest. Dilutions were made in phosphate buffer pH 7.3 to 7.4. Egg infectivity titrations (EID_{50}) and reciprocal hemagglutinin titrations (HA) were conducted by usual methods(5,6). Tissue cultures were inoculated with 10^3 to 10^4 EID_{50}. All data represent average values of 3 experiments. The 112th allantoic sac passage of strain WS was received through the courtesy of Dr. R. R. Wagner. The 114th allantoic sac passage of strain WS was used in all experiments. Strain WS showed no neurotropic properties on intracerebral inoculation of mice. Lyophilized 8th mouse brain passage of strain NWS was kindly provided by Dr. R. W. Schlesinger. The 3rd allantoic sac passage of strain NWS was used in all experiments. In 7 subsequent intracerebral passages in mice strain NWS produced a uniformly fatal disease. *Tissue cultures and substrate.* Minced and washed chorioallantoic membranes from 10- to 12-day-old chicken embryos were used in all experiments, as previously described(2). Balanced salt solution (BSS)(7) without glucose and $NaHCO_3$ was used as the basal solution. Reagent grade† carbohydrates, pyruvate, etc. were used. The final medium was adjusted to pH 7.6 with sterile NaOH before addition of tissue and virus.

Results. Table I summarizes data showing that chorioallantoic membrane tissue cultures (CamTC) maintained in 0.1% glucose supported multiplication of influenza virus

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† Nutritional Biochemical Corp., General Biochemicals, Inc., Schwarz, and California Foundation for Biochemical Research.

TABLE I. Multiplication of Strains WS and NWS in Tissue Cultures Provided with Pyruvate and with Glucose.

BSS plus:															
Pyruvate	0			.1%			0			0			.1 %		
Glucose	0			0			.1%			.02%			.02%		
	HA			HA			HA			HA			HA		
Virus (hr)	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
WS	<2	4	8	4	16	40	8	32	64	2	8	16	16	128	256
NWS	<2	<2	<2	<2	<2	<2	<2	32	64	<2	<2	±2	<2	128	128

BSS = Balanced salt solution without carbon source. HA = Reciprocal of avg viral hemagglutinin titers of 3 exp.

strains WS and NWS as measured by increases in HA, whereas in 0.1% pyruvate only strain WS multiplied. Cells in BSS without pyruvate or glucose supported multiplication of strain WS comparable with data obtained with strain PR-8 under similar conditions(1). CamTC in 0.02% glucose did not yield a significant increase in HA of strain NWS, however combination of 0.02% glucose and 0.1% pyruvate gave the highest HA observed for both viruses. In other experiments up to 5% pyruvate did not result in multiplication of strain NWS. In the presence of 0.1% pyruvate EID_{50} of tissue plus supernatant fluid determined at 12, 24, and 36 hours showed less than a 10-fold increase in infectivity of strain NWS, whereas increases of 1000-fold and greater were found with strain WS.

That the failure of CamTC to synthesize NWS virus, in presence of pyruvate, was not due to failure of virus to infect the cells, was shown in the following experiments similar to those previously reported in studies on potassium and infection with strain PR-8(2). Strain NWS at a titer of 10^3 EID_{50} was incubated with chorioallantoic membrane in the pyruvate medium for 2 hours and then anti-NWS rabbit serum was added. When the tissue was washed with physiological saline to remove the immune serum and fluids were replaced with 0.1% glucose medium, HA of 16 were detected after 72 hours. This result indicated that in the pyruvate medium strain NWS incubated with tissue became attached to cells (or penetrated cells) and was thus made insusceptible to neutralization by antibody.

The synergism observed with the combination of 0.02% glucose and 0.1% pyruvate suggests that glucose supplies factors essential for synthesis of strain NWS in CamTC. Compounds comparable to glycolytic products, Krebs cycle intermediates, high energy phosphates, coenzymes, etc. were tested for their influence on multiplication of strain NWS in CamTC. The data in Table II show that when CamTC was maintained in the presence of glucose, xylose, or glycerol, an increase in HA was observed. Ribose, dihydroxyacetone, glycerophosphate, adenosine-5

TABLE II. Effect of Various Substrates on Multiplication of Strain NWS in Tissue Cultures.

BSS + Substrate	.1% HA	.02% + .1% pyruvate HA
None	<2	
Na pyruvate	<2	
Glucose	32	128
D (+) Xylose	8	8
D (−) Ribose	<2	16
D (−) Arabinose	<2	<2
Dihydroxyacetone	<2	32
Glycerol	32	32
Na glycerophosphate	<2	8
NH_4 lactate	<2	<2
Na acetate	<2	<2
Adenine sulfate	<2	<2
Adenosine	<2	<2
" -5 phosphate	<2	32
" diphosphate	<2	32
" triphosphate	<2	32
Diphosphopyridine nucleotide	<2	64
Triphosphopyridine nucleotide	<2	4
Glutamine	<2	<2
Ca gluconate	<2	<2
Fumaric acid	<2	<2
Cis-aconitic acid	<2	<2
Isocitric acid	<2	<2
Malic acid	<2	<2
Succinic acid	<2	<2
Keto-glutaric acid	<2	<2
Oxalic acid	<2	<2

BSS = Balanced salt solution without carbon source.

HA = Reciprocal of avg viral hemagglutinin titers of 3 exp. after 48 hr.

phosphate, adenosine diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, or triphosphopyridine nucleotide in combination with pyruvate resulted in an increase in HA, though they were without effect when tested individually as the sole carbon source. Results with arabinose, lactate, acetate, adenine, adenosine, glutamine, gluconate, fumaric acid, cis-aconitic acid, isocitric acid, malic acid, succinic acid, ketoglutaric acid, or oxalic acid were negative when tested as a sole carbon source and in combination with pyruvate.

Discussion. The data in Tables I and II suggest that propagation of strain NWS in cells of the chorioallantoic membrane may require nucleotides or carbon sources not supplied by cells in the pyruvate medium, which nevertheless support growth of the parent strain. The difference in requirements between strains NWS and WS may or may not be a variation associated with neurotropism. This can only be determined after the growth of other influenza strains on CamTC in pyruvate and glucose has been studied. It is believed that with CamTC in a pyruvate medium adsorption and penetration of strain NWS is possible but viral synthesis is markedly reduced. Diphosphopyridine nucleotide (coenzyme I) with pyruvate in CamTC had a greater effect on virus multiplication than triphosphopyridine nucleotide (coenzyme II) which might suggest greater specificity for coenzyme I. Possibly a coenzyme I linked reaction is indicated.

Summary. Chorioallantoic membrane tissue cultures (CamTC) in balanced salt solution (BSS) with pyruvate as the sole carbon source supported synthesis of influenza virus (strain WS) but not its neurotropic variant (strain NWS). Various other substrates were studied alone and together with pyruvate for their effect on synthesis of strain NWS in chorioallantoic membrane. Cells maintained in BSS containing glucose, xylose, or glycerol as the sole carbon source supported viral synthesis, while cells maintained in ribose, dihydroxyacetone, glycerophosphate, adenosine-5 phosphate, adenosine diphosphate, adenosine triphosphate, or diphosphopyridine nucleotide supported viral synthesis only in combination with pyruvate. It is suggested that synthesis of strain NWS in CamTC requires nutritional factors which are not supplied by cells maintained in a medium with pyruvate as the sole carbon source.

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Role of Mammary Glands on Weight of Suckling Rats. (22717)

P. K. DIKSHIT AND A. D. TASKAR (Introduced by Arnold Lazarow)

Nutrition Research Laboratories, Indian Council of Medical Research, Coonoor, South India

In nutritional studies there is a great need for employing rats showing uniformity in weight. In the weanling rats reared in these Laboratories it has not been possible to obtain appreciable uniformity in weights. The individual variation was considerable although the average weaning weight of animals born during different periods remained fairly constant.* It is now known(1) that the weaning weight of rat is influenced by the following factors: (a) the number of young the mother suckles, (b) the position of the litter in litter series, (c) the nature of the diet. While studying these aspects, it was noticed that

* Unpublished data.

TABLE I. Relationship between "Functional" Mammary Glands and 14th Day Weight of Offsprings.

No. of functional mammary glands	8	9	10	11	12
No. of females	30	46	78	37	30
14th day wt of 6 young (g)	124.3	128.4	132.3	140.0	139.1
S.E.	2.69	2.93	2.10	2.88	2.99
Avg birth wt of 6 young (g)	29.91	30.11	30.16	30.30	30.37
S.E.	0.212	0.211	0.398	0.389	0.363

there was some relationship between the number of well developed mammary glands of the lactating female and the weaning weight of the young. The present communication attempts to show this relationship.

Material and method. Albino rat mothers from the stock colony between the ages of 5 and 12 months, each nursing 6 young ones, were randomly selected. On the 14th day, a careful examination of their mammary glands was carried out, and the number of teats being suckled by the young ones noted. Normally, a female rat possesses 6 pairs of teats, 3 in the thoracic and 3 in the abdominal region. The teats which contain milk project prominently due to frequent sucking by the young. It is possible to exude milk from these "active" or "functional" nipples when they are gently squeezed, while those not prominent appear to be completely devoid of milk. The milk containing glands are easily distinguishable from those not containing milk as the former besides projecting prominently are marked by a circular hairless area immediately around it. On the 14th day when the number of active mammary glands was counted the body weight of young also was recorded. The reason for weighing the young on the 14th day instead of on the weaning day, *viz.,* 21st day, is that up to this day the young are known to be entirely dependent upon the mother's milk(2). Any variation in their weights at this time could be taken as a criterion of the lactating capacity of the mother rat.

Results. Relation between number of mammary glands and weight of young rats: Table I shows that the total weight of 6 young on the 14th day increases correspondingly with the increase in the number of "active" mammary glands. This is found to be statistically significant. The trend can be

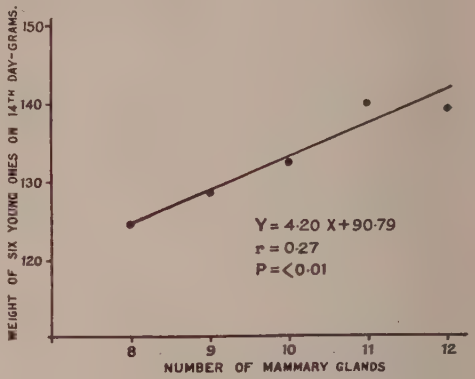


FIG. 1. "Functional" mammary glands and body wt of young.

best described by a linear regression $Y = 4.20 \times +90.79$, as depicted in Fig. 1. It is quite likely that the birthweight and the position of the litter series may to some extent determine the 14th day weights of young although the experimental evidence does not substantiate this, as is evident from Table I. This is what should be expected, since while the 14th day weight is dependent upon the available milk from the mother and probably the number of "functional" mammary glands, the average birth-weight is dependent upon the weight, general condition of the mother and the position of the litter series.

Number of mammary glands and position of the litter series: It has been observed in these Laboratories that the weaning weight of rats shows an increase with the order of birth. In order to investigate whether the above fact could be attributed to a corresponding increase in the number of functional glands, the data collected on the same 221 female rats were regrouped according to the order of birth, and summarized in Table II. It is clear from Table II that the average 14th day weight of the young born in the first lit-

TABLE II. Relationship between Litter Series, 14th Day Weight of Young and Number of "Functional" Mammary Glands.

Position of litter series	I	II	III	IV
No. of females	89	59	39	34
Avg No. of functional glands	9.6	10.2	10.3	10.2
S.E.	0.12	0.17	0.17	0.20
Avg wt of 6 young on 14th day (g)	122.5	137.5	143.1	138.3
S.E.	1.59	2.30	2.69	3.14
Avg birth wt of 6 young (g)	29.35	30.28	31.38	30.71
S.E.	0.223	0.173	0.696	0.243

ter was the lowest as compared to the weights of those of the subsequent series. The average 14th day weight of 6 young born in the third order of birth was the highest. It is interesting to note that the average number of "active" mammary glands in the females of the first group was the least. It may seem, therefore, that there is a correlation between the number of "active" mammary glands and the position of litter series.

Discussion. The wide variation in the weights of young rats from different litters has been mainly due to the maternal milk supply. A closer study revealed that there was a linear relationship between the number of "milking" mammary glands of the mother rats and weights of their offspring. Actually it is possible to explain on the basis of the number of "active" or "milking" mammary glands the observed differences in the weaning weights of young born in the different order of birth. It should, however, be admitted that a corresponding study of milk yield of the lactating mothers would have been of considerable importance, but certain technical difficulties did not permit of extensive studies being made on this aspect.

Summary. An attempt has been made to determine the cause(s) for variation in weight of suckling rats. It was found that the 14th day weight of rats did depend upon the number of "milking" glands of the mother rats. The average 14th day weight of young born in the first litter was lowest when compared with the weights at corresponding periods of those delivered in the subsequent litters. The average number of "functional" mammary glands of mothers nursing their first born was also the lowest as compared with those of mothers rearing their second, third and fourth litters.

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Essential Fatty Acids in the Chick. I. Development of Fat Deficiency.* (22718)

J. G. BIERI, G. M. BRIGGS, M. R. SPIVEY FOX, C. J. POLLARD, AND L. O. ORTIZ
Laboratory of Nutrition and Endocrinology, N.I.H., P.H.S., Bethesda, Md.

Symptoms of essential fatty acid deficiency in various animal species in which it has been produced are rather similar. Thus, in the rat(1), dog(2), guinea pig(3), and mouse (4), onset of deficiency is relatively slow and becomes apparent in changes in skin and fur as well as in decreased growth rate. The periods required for depletion of essential fatty

* Preliminary report appeared in *Poultry Sci.*, 1955, v34, 1231.

TABLE I. Composition of Purified Diet.

Ingredient	g/kg
Purified casein*	200
Gelatin	80
Glucose (cerelose)	615
Corn oil†	40
DL-methionine	3
Salts A(6)	60
Vitamins‡	2
Aureomycin§	

* General Biochemicals, Inc.

† Mazola.

‡ Vitamins added as follows (in cerelose premix), mg/kg diet: Vit. B_1 8, vit. B_2 8, calcium pantothenate 20, choline chloride 2000, niacin 100, vit. B_6 8, d-biotin 0.3, folic acid 3, vit. B_{12} 0.02, vit. D_3 0.02, α-tocopherol acetate 100, and 2-methyl-1,4-naphthoquinone 1. Vit. D, E, and K added in ethanol. 30 mg/kg of vit. A acetate added in ethanol in first exp. 1 mg vit. A acetate given orally, weekly, in 2nd and 3rd exp. See text.

§ 200 mg/kg diet added after 8 wk of 2nd and 3rd exp.

acids, as detected by these symptoms, are 4 weeks in the mouse and guinea pig, 8-12 weeks in the rat, and 12-40 weeks in the dog. Morbidity and death in these animals occur considerably later. It is apparent that tissues of these species relinquish their polyunsaturated fatty acids at a slow rate. In contrast, it has been reported that chicks on a synthetic-type, fat-free diet grew slowly from the first week, feathered poorly, and died within 3 to 4 weeks(5). These observations suggest that the chick has a considerably greater essential fatty acid requirement than other species. Contrary to this viewpoint, our studies indicate that chicks grow normally on a fat-free diet for at least 6 to 8 weeks, and do not develop symptoms of deficiency until later.

Methods. Day-old female New Hampshire chicks in groups of 6 were placed in electrically heated brooders with screen mesh floors and fed the synthetic-type diet given in Table I, with or without inclusion of 4% corn oil. Both feed and water were given *ad lib.* The air-conditioned room was kept at 75 $\pm$ 1° F and 55% relative humidity. In initial experiments it was found that 3 mg of vit. A acetate/kg, mixed into the diet in ethanol, was so rapidly destroyed (as found by analysis) that vit. A deficiency frequently developed in the chicks in 3-4 weeks. This could be prevented by increasing the level to 30 mg/kg. However, occasional appearance of vit. A deficiency symptoms even under these conditions prompted a study of parenteral and oral administration of this vitamin. A single, weekly oral dose of 1 mg vit. A acetate in 0.4 ml of 20% aqueous Tween 40† gave a sufficiently high liver storage of the vit. to prevent deficiency. Chemical analyses of livers from chicks in these experiments showed that both vits. A and E were absorbed in adequate amounts. Chicks were weighed and examined weekly. When 8 weeks old, the groups were reduced to four birds each because of space limitations.

Results. In preliminary experiments lasting 4 weeks, birds on fat-free diet grew as well as those receiving 4% corn oil in the diet and appeared normal in all respects as indicated in a preliminary report(7). Experiments of longer duration were then carried out and growth data from 3 separate trials are averaged in Table II. Although control chicks were slightly heavier throughout, the difference at 8 weeks was not significant ($P = >0.1$). By 12 weeks chicks fed the fat-free diet were definitely smaller ($P = <0.01$). It was apparent from the growth curves that effects of fat-deficiency appeared between the 6th and 8th weeks. The first visible symptom attributed to fat deficit was a lightening of normally dark brown plummage, occurring from about the 8th week. This was not marked and did not progress or occur uniformly in all birds. Changes in skin were not readily detectable. In several birds an increased scaliness occurred after 16-20 weeks.

Of the 8 fat-deficient chickens kept beyond 8 weeks, 4 were maintained as long as 24 weeks. Three others died at 13, 22 and 24 weeks, and one was killed at 17 weeks. Throughout this period the appearance of the birds was normal except for the slight changes noted above. Death of chicks in the first trial was attributed to air sac infection. In succeeding experiments 200 mg aureomycin/kg of diet was fed after the birds were 8 weeks of age and this cause of death was eliminated.

† Polyoxyethylene sorbitanmonopalmitate.

TABLE II. Growth of Chicks Fed a Fat-Free Diet (Avg of 3 Trials).*

	Fat-free diet			Control diet		
Age (wk)	Wt in g	No. surviving	Feed effic.†	Wt in g	No. surviving	Feed effic.
4	295 ± 11‡	18	.549	303 ± 12	18	.537
6	560 ± 13	17		579 ± 16	18	
8	830 ± 26	17		894 ± 30	16	
12	1288 ± 46	8*		1512 ± 49	8*	

* Six chicks/group in each trial. Only 2 trials were continued for 12 wk and longer; the No. of chicks was reduced to 4 in each group after 8 wk.
† Total wt gain/total food consumed.
‡ Mean ± S.E.

In two birds which died in later experiments, no obvious cause of death could be detected. At autopsy, fat-deficient chickens (24 weeks) had considerable amounts of abdominal fat, though less than controls. The oviducts and ovaries in two birds were immature, but in the other two the ovaries contained developed yolks. All other organs appeared normal.

Three male chicks maintained on fat-free diet for 8 weeks had combs and wattles which were smaller and paler than those of three males receiving 4% corn oil in the diet. At autopsy, the testes of fat-deficient chicks were only half as large as those of control chicks (178 vs. 441 mg/kg body weight). Body weights of deficient birds were only slightly less (6%) than those of normal birds.

Water consumption measurements made during the ninth week revealed that fat-deficient birds drank only about 15% more than the controls. In two trials made during the 11th and 12th weeks deficient chicks consumed 137 ml/kg/day compared with 103 ml/kg/day for normal chicks (4 per group).

Discussion. These experiments show that when chicks are fed a synthetic-type diet devoid of fat but adequate with respect to all other nutrients, growth continues normally for at least six weeks. Furthermore, the symptoms of fat deficiency that eventually develop are not striking. These results are in marked contrast to the picture presented by Reiser(5), and which has been quoted in recent reviews(8,9), that chicks grow poorly and die within one month when deprived of fat. It would appear that the diet used by Reiser was probably deficient in nutrients other than fat. The difficulty in insuring a sufficient intake of vit. A in a fat-free diet has already been discussed above. In addition to a possible vit. A deficiency, the diet of Reiser may also have been low in thiamine since the soybean protein, used in his diets, contains sulfite which has been shown to destroy thiamine(10). The salts V, which he used, accelerate thiamine destruction(11). Repetition of Reiser's experiments in our laboratory, using egg albumen as the protein but with vit. A given orally as described above, did not result in any difference between control and fat-free birds at 4 weeks

Analysis of purified casein(12) used in these experiments revealed a fat content of 0.23% and examination of the polyunsaturated fatty acids(13) in this lipid showed only 4.4% of dienoic and no trienoic or tetraenoic acid. This would amount to an intake of less than one mg dienoic acid daily (0.046% lipid in the diet). In comparison with the linoleic acid requirement of other species, this amount would be negligible. This same casein in a similar purified diet readily produces essential fatty acid deficiency in the guinea pig(3).

The decrease in growth rate, which occurred after 6-8 weeks on the fat-free diet, was also found by Russell *et al.*(14), although their difference began at 10 weeks and was not as marked. The higher caloric content, or an increased palatability of the control diet with fat, may have contributed to these differences. In the present studies feed efficiencies of the control and fat-free birds were found to be similar at 4 weeks (Table II).

Summary. Day-old chicks raised on a purified diet containing approximately

0.046% fat grew normally for 6-8 weeks, at which time weight gains began to decrease slightly. No marked symptoms of deficiency developed, but depigmentation of feathers and scaliness of skin were observed. Fat-deficient male chicks had smaller testes and some females had immature oviducts, when compared with normal chicks. Water consumption by the deficient birds did not increase markedly until the 11th week on the fat-free diet.

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Variations in After-Discharge from the Hippocampus. (22719)

R. E. CORRELL AND W. R. INGRAM

Departments of Psychiatry and Anatomy, University of Iowa

A number of investigators have noted the relative sensitivity of the hippocampal formation to the production of after-discharge(2,5, 8,9). It has also been noted that the after-discharge shows little or no spread to the rostral neocortex(3), although there is some disagreement on the extent of spread. Akert and Andy(1) describe the local after-discharge in the guinea pig as characterized by 18-25 c/sec. regular waves with occasional activity at slower or faster frequencies. Liberson and Akert(6) describe a tonic-clonic sequence. The tonic phase consists of 10-30 c/sec. activity associated with brief spikes of 100-250 c/sec. frequency. The relation of the DC potentials, described earlier by Liberson and Cadilhac(7), to the clonic phase was indicated. Crutzfeldt and Meyer-Mickelett(3), using cats, describe 3 characteristic phases preceded by a period of 50-100 c/sec. activity. The first phase consists of 8-12 c/sec. waves and is followed by 18-28 c/sec. activity. The final phase consists of short pauses and single spikes.

The purposes of the present study were to determine the components of hippocampal after-discharge in the cat, and more specifically the different patterns which these components may form. A study was also made of variations in after-discharge threshold within the hippocampal formation, and an attempt was made to relate variations in pattern of after-discharge to a number of antecedent conditions.

Material and methods. Bilateral bipolar electrode placements were made in a series of 20 cats with the aid of a Horsley-Clark stereotaxic instrument. All operations were performed under aseptic conditions using sodium pentobarbital (Nembutal) anesthesia. In all cases placements were as nearly symmetric as possible, and at the level of the posterior portion of the lateral geniculate body. Some variation was introduced in the vertical plane

to cover different structures within the hippocampal formation. The bipolar electrodes were nichrome steel, insulated to within one millimeter of the tips, and placed with the tips one-half to one millimeter apart. Small stainless steel screws were placed in the skull over the middle suprasylvian gyrus to serve as cortical recording electrodes. All recording was done with a Grass Model III-D Electroencephalograph. Stimulation was by means of a Grass Model 3C Stimulator and isolation unit. Stimulation was with diphasic pulses of one millisecond duration at a rate of 80 pulses per second (pps.). To study the effects of stimulus frequency rates of 20 pps. and 300 pps. were also used. No records were taken for several days following operation to allow complete recovery of the animal. After-discharge was produced by stimulating the hippocampal formation with the stimulus parameters described for 10 seconds at intensities of 1 to 5 volts. In studies on effects of voltage the duration of stimulus was reduced to 5 seconds to minimize tissue destruction at high voltages. The duration of the after-discharge periods ranged from 2 or 3 seconds following termination of stimulus to just under 2 minutes. Most of them lasted between 30 and 40 seconds. For analysis of the patterns of after-discharge, only those which persisted long enough for development of a clear pattern were used.

Analysis of the after-discharge activity is based on 91 seizure records of 16 cats. Studies of the effects of voltage, pulse rate, time between seizures and mode of stimulation (ipsilateral, bilateral or contralateral) were carried out on 4 additional cats. The right hippocampal tracing was the one analyzed in all cases. For bilateral stimulation the right and left hippocampal bipolar electrodes were connected in parallel. Voltage was monitored at all times by means of a cathode ray oscilloscope. Histological control was available for all animals.

Results. The most frequently seen components were spikes of varying duration. They tended to fall in bands of 15-20 milliseconds, 30-40 milliseconds and 60-80 milliseconds. Occasionally faster spikes were visible, but their characteristics could not be adequately determined with an inkwriter. The slower spikes were seen more frequently than the faster and ranged in amplitude from 1 to 2 millivolts. The faster spikes were usually at lower voltage. These faster spikes typically were diphasic or triphasic in form and characteristically ranged from 100 to 600 microvolts in amplitude. Slower waves, more sinusoidal in form, were seen at a frequency of 5-11 c/sec., and occasionally activity at ½ to 4 c/sec. was present. Rhythmic activity between 20 and 30 c/sec. was at times seen for brief periods. It was most often present at 26 c/sec., but also frequently at 22 c/sec.

These components tend to become organized into several relatively discrete patterns. Five such patterns were empirically defined on the basis of wave form, frequency and voltage of the predominant activity. Examples of these groups are shown in Fig. 1. Group 1 was defined on the basis of a low to moderate voltage (100 to 400 microvolts peak to peak) 1½ to 9 c/sec. rhythmic activity. The slower activity was usually at the lower voltages. This activity may occur alone, but often the shift across the baseline is accompanied by a burst of fast low voltage spikes. Occasionally this activity may occur with a near normal tracing superimposed on it. In some records the spikes bear a constant relationship to the slower wave, but often they do not. Rarely a classical "wave and spike" pattern was seen.

Group 2 was the largest group, and 12 of the 16 animals had records falling in this group. It includes those cases where the predominant activity is spikes. The slower spikes were more highly organized and occurred at a repetition rate of 7½ to 12 per second, but most frequently at 9 per second. These spikes are monophasic and occur on the crest of a low voltage (50 to 100 microvolts) slow wave. The faster spikes are more complex, as previously indicated, and also occur with slower waves of low voltage and rhythmic 20 to 30 c/sec. activity. These records tend to show more components with more variable relationships between them than do the records with slower spikes.

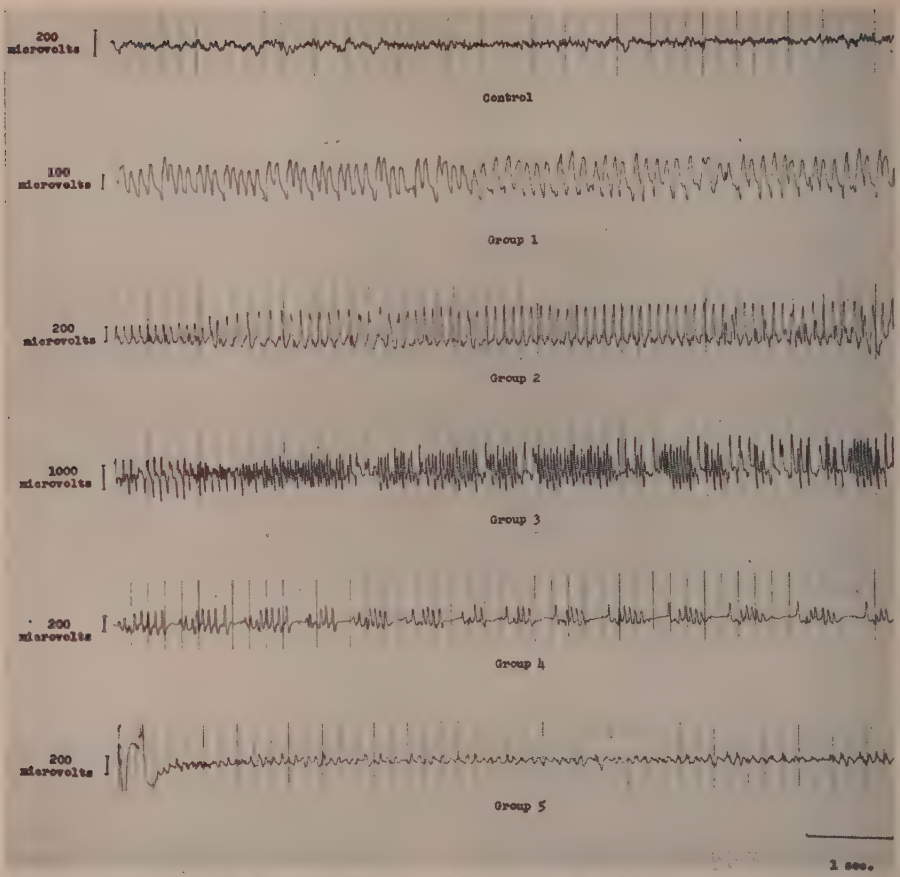


FIG. 1. Patterns of after-discharge obtained by stimulation of the hippocampal formation, and a representative section of normal hippocampal activity. These records were taken from different cats.

Group 3 is relatively rare, and was seen in only 3 of the 16 animals. It was defined on the basis of a predominant rhythmic fast activity ranging from 20 to 30 c/sec., but most often seen at 26 c/sec. Sharp spikes and slow waves are the most usual accompanying activity. Group 4 was defined on the basis of an alternating burst and depression. Most typically the bursts are made up of 4 or 5 15-30 c/sec. sharp waves which are separated by a silent period. This pattern was seen in 7 of the 16 cats. Group 5, seen in 5 of the 16 cats, was defined on the basis of a low voltage continuous rhythmic activity at frequencies of 5-15 c/sec. This may be mixed with low voltage fast spikes. More than one of these patterns may appear during a single seizure, though one is nearly always predominant. If the records are grouped on the basis of the predominant pattern, records from the same cat fall into several groups. The range was from 1 to 4 with a median of 2.5.

Fig. 2 illustrates a number of the characteristics of hippocampal after-discharge when the entire period of after-discharge is considered. When the after-discharge is compared with the control record, the absence of any great disturbance in the dorsolateral cortex is apparent. The pattern of after-discharge on the right and the left shifts markedly (more than is typical) throughout the course of the seizure, and the activity on

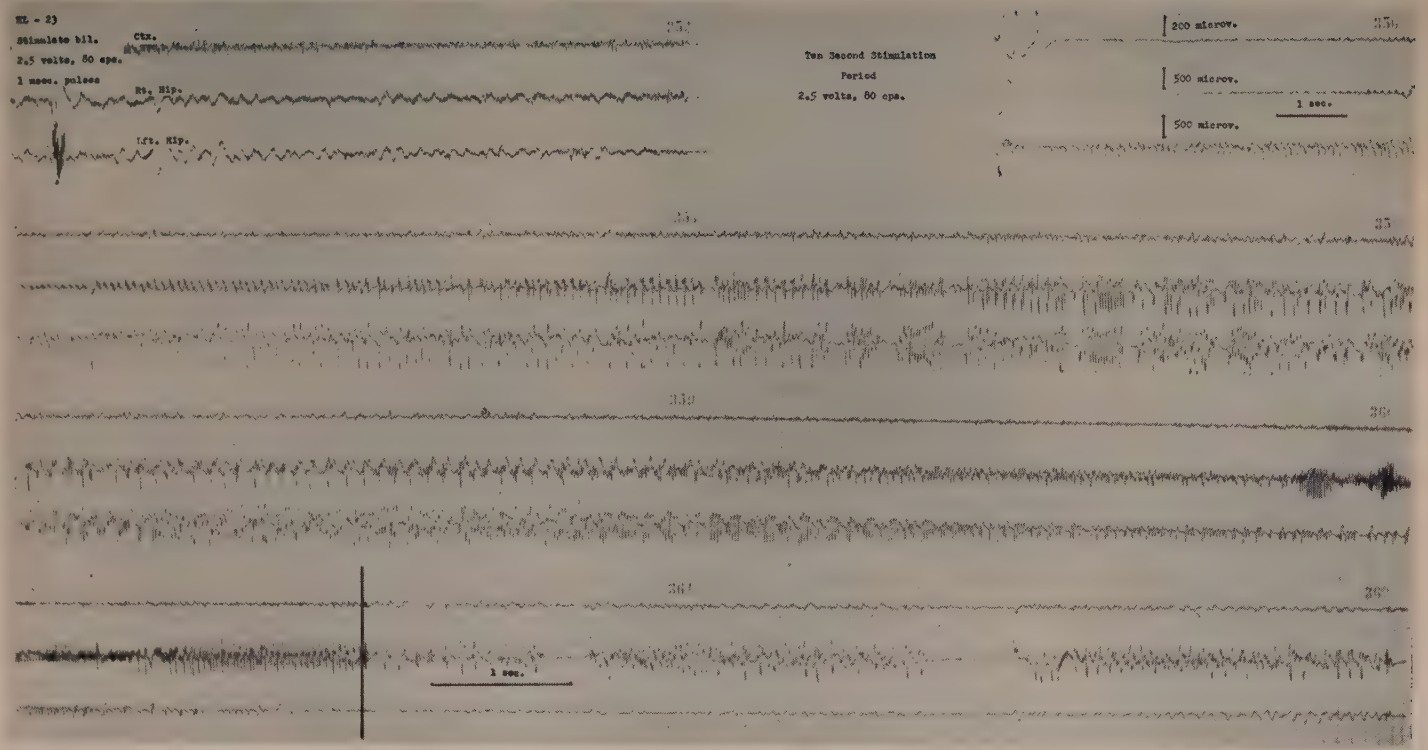


FIG. 2. A complete record of hippocampal after-discharge obtained by bilateral stimulation at 2.5 volts, 80 pps. See text for discussion.

the two sides is almost never identical. However, the brief silent periods, when they occur, usually occur simultaneously on the right and the left. Shifts in after-discharge form nearly always occur simultaneously in the 2 hippocampi. The long build-up which is present on the right is frequently seen, and may not be equal bilaterally. The gradual ending of the seizure activity on the left is atypical, as the seizures usually end quite abruptly and simultaneously on both sides. If there is a difference in the voltage of the after-discharge on the right and the left, the higher voltage is usually on the side stimulated. There is usually a brief period of depressed electrical activity following a seizure. In rare instances random spikes persisted for several minutes following a seizure, and appeared independently on the right and the left.

Of the locations investigated, the dentate fascia had the lowest thresholds for the production of after-discharge and the subiculum the highest. In some cases after-discharge could not be elicited from the subiculum. The alveus also has a high threshold in the only 2 placements in that location, as did the ventromedial edge of the hippocampus. In general, the fiber tracts running between the pyriform and retrosplenial areas and the hippocampus yielded an intermediate threshold.

Since the pattern of after-discharge was not dependent on the location of the electrodes, at least entirely, other factors were investigated. When the patterns were ranked by mean threshold and by mean time since operation there was a statistically significant relationship between the rankings ($r = .77$). Threshold means were computed by finding the overall mean for each cat and giving each record a value in terms of deviation from this mean. These deviation values were then averaged for each pattern. Group 4 showed the lowest threshold and Group 2 the highest. There was very little difference between the stimulus intensities required to produce after-discharge at 20, 80 and 300 pps., but 80 pps. seemed slightly more effective (not statistically significant). There was no evidence that any of the components of after-discharge, or the patterns they formed was related to the stimulus frequency, or to the voltage used provided it was above threshold. There was consistent evidence of reduced excitability two minutes following the end of a seizure. Occasionally longer intervals were tested, but the results were inconsistent.

Discussion. The results disclose considerably more variability in the form of the after-discharge from the hippocampal formation than has previously been indicated, although the components seen confirm those previously reported for the guinea pig(1,5) and the cat (4,8). The sequences of activity reported by Liberson and Akert(6), and by Crutzfeldt and Meyer-Mickelett(3) were both seen, but were no more frequent than other sequences noted. The complete tonic-clonic sequence was rather rare. The gradual build-up frequently seen would seem to indicate a recruitment process within the hippocampus. The fact that quite different patterns of after-discharge could be recorded from the same electrode implant with identical stimulus parameters would strongly suggest that the nature of the recorded activity is dependent upon the state of the organism at the time the stimulus is applied.

Attempts to determine the preceding conditions responsible for the variations in form of the after-discharge have not been very successful. The fact that there was a relationship between threshold and pattern suggests that the level of excitability at the time the stimulus is applied may be one significant variable. This is further supported by the significant relationship between threshold and time since operation for the different patterns, and would suggest some progressive modification of the hippocampus as one determining variable. If elements within the hippocampus are differentially affected by previous stimulation, then certain variations in pattern could represent the absence of participation of some elements. Additional work is necessary on this aspect.

Conclusions. The after-discharge of the hippocampus is formed by a number of components which can combine to form several fairly discrete well organized patterns. These

patterns seem to be related more to the state of the organism at the moment the after-discharge is produced than to the precise anatomic location of the electrodes or to the parameters of stimulation. Although the activity emanating from symmetrical electrode placements may show differences in pattern, there are systematic relationships between the right and the left which would indicate some bilaterally expressed control on the after-discharge. Extremely high voltage electrical after-discharge may be present in the hippocampal formation with only minimal disturbance in the dorsolateral cortex, at least by transverse recording, and without the precipitation of a major convulsion.

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The Effect of Molybdenum on Chick and Poult Growth. (22720)

B. L. REID, A. A. KURNICK,* R. L. SVACHA, AND J. R. COUCH
Departments of Poultry Husbandry and Biochemistry and Nutrition, Texas A. and M. College System, College Station, Texas

The ash of unidentified factor sources has been reported to stimulate the growth of both chicks and poults when supplemented to a purified type diet(1,2). The ash of dried whey, fish solubles or distillers dried solubles has also been found to stimulate the growth of chicks fed a practical type diet under field conditions(3).

Single additions of 40 elements to a purified diet for chicks were ineffective in duplicating the growth response obtained with the ash of a mixture of 6 unidentified factor sources(8). A reconstituted ash mixture, which was made up of chemically pure, reagent grade chemicals and supplied the elements found in the ash of distillers dried solubles upon spectrographic analysis, stimulated the growth rate of both chicks and poults (4). A similar reconstituted ash mixture produced a growth response under practical conditions (3).

Molybdenum has been found to be a co-factor of xanthine oxidase in the rat(5,6). The maximum molybdenum required by the rat for xanthine oxidase activity has been estimated as 0.2-0.3 μg per day(7). However, the growth rate of rats receiving the diets low in molybdenum was not affected. Molybdenum deficiency in the chick was associated with the feeding of 4.5-9.4 mg % tungsten in the ration(9). Such a deficiency could be overcome through addition of molybdate to the diet. The deficiency was characterized by growth depression, mortality, lowered tissue molybdenum concentrations, reduced xanthine dehydrogenase activity in the liver, intestine, kidney and pancreas, and decreased uric acid excretion.

Methods. Chicks were obtained from dams which had been reared from one day of age on an all-vegetable protein diet deficient in unidentified growth factors. The maternal diet used was the same as previously reported by Dannenburg *et al.*(1). Two types of chicks were used, New Hampshire and a New Hampshire X Barred Plymouth Rock Cross.

* Predoctoral Research Fellow of National Cancer Institute, N.I.H., P.H.S., Bethesda, Md.

TABLE I. Basal Diets.

Ingredients	Chicks	Poults
	g/kg	
Cerelose	625.7	528.8
Soybean protein (Drackett C-1)	250	325
Soybean oil	30	35
$CaCO_3$	14.5	16.03
$CaHPO_4 \cdot 2H_2O$	42.1	60.10
NaCl	9.5	5
$MnSO_4 \cdot 1H_2O$	.78	.81
$FeC_6H_6O_7 \cdot 5H_2O$	1.6	1.60
$CuSO_4 \cdot 5H_2O$	.016	.02
$ZnCl_2$	.014	.014
KI	.040	.040
$CoCl_2 \cdot 6H_2O$	.0005	.0005
KCl	5.72	6
$MgSO_4 \cdot 7H_2O$	5.7	5.76
	Amt/kg	
Thiamin HCl	4 mg	5 mg
Riboflavin	6	8
D-Calcium pantothenate	15	16
Pyridoxine HCl	4	4
Para-Aminobenzoic acid	20	20
Alpha-tocopheryl acetate	24	24
Inositol	1,000	1,000
Menadione	.5	.5
Biotin	.2	.2
Niacin	100	120
Choline chloride	2,000	2,000
Folic acid	2	2
Aureomycin HCl	10	50
Vitamin A	10,000 I.U.	10,000 I.U.
" D_3	2,000 I.C.U.	2,000 I.C.U.
" B_{12}	30 μg	50 μg
DPPD	125 mg	125 mg
DL-methionine	7.5 g	7.5 g
Glycine	4 g	4 g

The Broadbreasted Bronze poults were obtained from dams which had not been depleted of unidentified factors. The birds in each experiment were distributed at random into groups of 10 to 16 birds each, and in the case of the chicks each treatment was replicated once. Four replicates were used on the basal ration in the chick studies and two in the poult studies conducted. The birds were maintained in batteries with raised wire floors throughout the four-week experimental periods. Feed and water were supplied *ad lib.*

The basal diets used are shown in Table I. The diets for both the chicks and poults have been checked for adequacy by feeding higher protein levels, a combination of the 11 essential amino acids at levels of 0.1 of the N.R.C. recommended levels, additional vitamins as well as double and four times the trace minerals without producing additional growth. The soybean protein (Drackett C-1) was extracted with either 70% isopropanol or hot water (pH 4.6) in chick diets in order to remove any unknown growth factors associated with this dietary component. The ash of distillers dried solubles was prepared by ignition over an open flame followed by ashing in a muffle furnace at 600°C until a constant weight was obtained (8 to 10 hours). The composition of the reconstituted ash mixture used which consisted of the elements present in the ash upon spectrographic analysis is shown in Table II.

Results. Feeding molybdenum (0.0126 ppm) in a purified diet for chicks resulted in an 18.7% increase in growth (Exp. 1, Table III). In the same experiment, distillers dried solubles produced an increase in growth of chicks to 4 weeks of 97 g over the unsupplemented group. Approximately one-half of the growth response obtained with distillers dried solubles was produced by feeding the ash of this product at a level equivalent to 3%. The reconstituted ash mixture was equivalent to distillers dried solubles ash in growth-promoting properties (Table III).

In the study using crossbred chicks, a 13.5% growth increase was obtained through addition of 0.0126 ppm of molybdenum to the basal diet. This response was found to be significant at the 0.05 level of probability. The reconstituted ash mixture in this experiment produced an 11.4% increase in growth while the higher level of molybdenum (3.3 ppm) produced a growth response of 9.7%. Both of these growth rates were significantly greater than that of the unsupplemented control group (Table III).

Using Broadbreasted Bronze turkey poults, a 29.1% growth response was obtained with reconstituted ash mixture fed at a level equivalent to 6% distillers dried solubles. Inclusion of 0.0126 ppm of molybdenum in the basal diet produced a 19.1% increase in growth rate.

It is interesting to note that growth responses obtained with molybdenum in these

TABLE II. Composition of Reconstituted Ash Mixture.

Ingredient	Formula	Amt equivalent to 100 g ash* mg	g
Aluminum sulfate	$Al_2(SO_4)_3 \cdot 18\ H_2O$	370.8	
Barium hydroxide	$Ba(OH)_2 \cdot 8H_2O$	13.7	
Sodium borate	$Na_2B_4O_7 \cdot 10H_2O$	264.0	
Chromic oxide	Cr_2O_3	1.46	
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	4.04	
Copper sulfate	$CuSO_4$ (anhydrous)		3.93
Ferric phosphate	$FePO_4 \cdot 4H_2O$		12.0
Magnesium sulfate	$MgSO_4$ (anhydrous)		3.04
Manganese chloride	$MnCl_2 \cdot 4H_2O$		1.08
Molybdic acid	$H_2MoO_4 \cdot H_2O$	56.26	
Nickelous carbonate	$NiCO_3 \cdot 2Ni(OH)_2 \cdot 4H_2O$	64.08	
Ca. phosphate (dibasic)	$CaHPO_4 \cdot 2H_2O$		7.33
Potassium chloride	KCl		5.73
Sodium meta-silicate	$Na_2SiO_3 \cdot 9H_2O$		3.04
Sodium chloride	NaCl		7.63
Strontium chloride	$SrCl_2 \cdot 6H_2O$	304	
Titanium oxide	TiO_2	5	
Zinc chloride	$ZnCl_2$	62.53	
Lead nitrate	$Pb(NO_3)_2$	159	
Silver nitrate	$AgNO_3$	47.2	

* 451.3 mg of reconstituted ash mixture were equivalent to 1 g of distillers dried solubles ash.

studies were equivalent to those obtained with either distillers dried solubles ash or reconstituted ash mixture in the chick experiments, while the results of the poult study indicate an added growth advantage through the feeding of reconstituted ash at a higher level. These data would indicate a higher requirement for molybdenum in the turkey poult under the conditions of these studies. The molybdenum contents were determined to be 0.972 ppm and 1.58 ppm, respectively, for the chick and poult diets. It is difficult to understand how inclusion of only 0.0126 ppm of molybdenum in these diets would produce an increase in growth, unless a large portion of the molybdenum present in the basal rations was unavailable. Richert and Westerfeld(7) have indicated that the molybdenum in soybean oil meal was largely unavailable to the rat. The molybdenum content of soybean protein (Drackett C-1), before alcohol or water extraction, was found to be 4.77 ppm; while the extracted protein contained 3.84 ppm of molybdenum. If it were assumed that

TABLE III. Effect of Minerals on Chick Growth.

Supplements to basal diet	Exp. 1 (New Hampshire chicks) 4-wk avg wt (g)	Exp. 1 Response over basal (%)	Exp. 2 (New Hampshire × Barred Rock) 4-wk avg wt (g)	Exp. 2 Response over basal (%)	Exp. 3* (Broadbreasted Bronze poults) 4-wk avg wt (g)	Exp. 3 Response over basal (%)
None	289.7	—	346.1	—	403	—
3% distillers dried solubles	376.7‡	26.2	—	—	—	—
Distillers dried solubles ash equivalent to 3%	330.3†	14.0	—	—	—	—
Reconstituted ash equivalent to 3% distillers dried solubles	335.2†	15.7	385.6†	11.4	520‡	29.1
Molybdic acid, 0.0126 ppm Mo	344.0†	18.7	399.9†	13.5	480†	19.1
" " 3.3 " "	—	—	379.8†	9.7	—	—

* The reconstituted ash was fed at level equivalent to 6% Distillers Dried Solubles.
† Significant at 0.05 level of probability.
‡ " " 0.01 " " " .

molybdenum in the protein source was unavailable to the chick, the basal diet would contain only 0.0228 ppm of "available" molybdenum supplied by the mineral mixture. However, this remains to be proven. The turkey mineral mixture contained 0.3 ppm of molybdenum and supplied 0.0287 ppm to the basal ration. It would appear that both the chick and poult have a nutritional requirement for molybdenum under the conditions of these studies.

Summary. 1) The results of 2 studies with chicks and one study using Broad Breasted Bronze turkey poults have indicated that addition of molybdenum to purified basal diets resulted in a growth advantage of 13.5-19.1% over the unsupplemented group. 2) The feeding of a reconstituted ash mixture which simulated the ash of distillers dried solubles resulted in significant increases in growth rate of both chicks and poults. 3) From the data presented it appears that under conditions of these studies a nutritional requirement for molybdenum by chick and poult may be demonstrated.

This work was supported in part by a grant-in-aid from the Robert A. Welch Foundation, Houston, Texas. The following products were supplied through the courtesies as indicated: Inositol and Cerelose (monohydrate), Corn Products Refining Co., Argo, Ill., folic acid and aureomycin, Lederle Laboratories Division, Amer. Cyanamid Co., Pearl River, N. Y., biotin, Hoffmann-LaRoche, Inc., Nutley, N. J.; soybean oil, the Buckeye Cellulose Corp., Cincinnati, O.; methionine and glycine, Dow Chemical Co., Freeport, Texas; stabilized vit. A, Stabilized Vitamins, Inc., New York; vit D_3, Bowman Feed Products Co., Holland, Mich.; diphenyl-paraphenylenediamine, B. F. Goodrich Chemical Co., Cleveland, O.; alpha tocopheryl acetate, menadione, riboflavin, thiamin HCl, D-calcium pantothenate, para-aminobenzoic acid, pyridoxine HCl, niacin, choline chloride and vit B_{12}, Merck & Co., Rahway, N. J.

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4,4′-Bisacetamidocarbanilide, a Metabolite of Nicarbazin. (22721)

A. C. CUCKLER, H. D. BROWN, AND H. SIEGEL (Introduced by H. J. Robinson)

Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, Rahway, N. J., and Albert Einstein College of Medicine, New York City

Nicarbazin, a chemical complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4, 6-dimethyl pyrimidine (HDP), is an effective and widely used anticoccidial agent for chickens(1,2). After nicarbazin is absorbed by chickens, the complex separates into its components, which are rapidly eliminated from the tissues(3). Clark, *et al.*(4) used nicarbazin labelled with C^{14} in the benzene ring of the DNC to determine if metabolites, which were undetected by chemical assay, remained in the tissues. These studies showed that most radioactivity found in tissues of chickens fed or dosed with C^{14} labelled nicarbazin was accounted for as colorimetrically assayable DNC. Although traces of metabolites of DNC were present in tissues of chickens, they were rapidly destroyed or excreted.

During the performance of tolerance studies with large doses of nicarbazin(5),

crystalline deposits were noted in the renal tubules of calves. Therefore, studies were conducted to isolate this material, which was identified as 4,4′-bisacetamidocarbanilide, a metabolite of nicarbazin.

Methods. Nicarbazin was fed to 8-10-week-old calves for 4 to 6 weeks. The nicarbazin, in daily dosages of 0.04, 0.2 or 1.0 g/kg, was suspended in about one-half of the calves' morning milk ration. When this was consumed, the balance of the milk was fed. Water, hay and a commercial calf-starter were fed *ad lib*.

Results. The calf on the lowest dosage of nicarbazin (0.04 g/kg) consumed 93 g of the drug during 6 weeks of administration. This calf gained in weight from 108 to 167 lb, appeared normal and maintained a good appetite. When this calf was sacrificed at end of dosage period, all organs were grossly and histologically normal. Another calf was fed a total of 565 g of nicarbazin in a daily dosage of 0.2 g/kg. During the first week of administration, this calf lost 10 lb in weight but afterwards gained slowly, increasing in weight from 153 to 183 lb. Although this calf continued to have a good appetite and appeared normal clinically, at autopsy the kidneys were enlarged and showed evidence of damage. The calf fed nicarbazin at the rate of 1 g/kg received 1524 g of the compound. Failure to gain in weight was evident during the first week of administration. During the next 3 weeks this calf lost 34 lb in weight, refused to eat normally, weakened and died. Necropsy examination showed the kidneys were enlarged. The cortex was pale and firm. The medulla revealed gray streaks next to the cortex and in its inner portion there were groups of minute tan-colored foci suggestive of crystalline deposits.

Histopathological examinations were made on the myocardium, aorta, bone marrow, lymph nodes, thymus, thyroid, lungs, pancreas, stomach, intestines, urinary bladder, adrenals, striated muscle, liver and kidneys. The only significant pathologic alterations were in the liver and kidney. The liver lesion was found only in the calf fed the highest dosage of nicarbazin (1 g/kg). The lesion

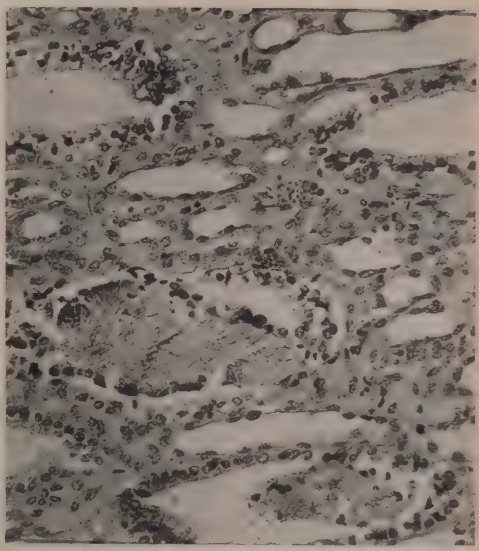


FIG. 1. Crystals of 4,4′-bisacetamidocarbanilide in collecting tubules of calf kidney.

was confined to the portal area and consisted of a slight to moderate bile duct proliferation and fibrosis. The kidney changes included precipitation of crystals (Fig. 1) mainly in the distal portions of the collecting tubules and a few in the convoluted tubules, probably the distal ones. The tubular epithelium directly adjacent to the crystals showed hyperplastic changes consistent with a foreign body reaction. The cortex was characterized by a focal, granulomatous, interstitial reaction. Within these areas of the cortex, hyperplastic changes were present in the tubules and small blood vessels, even in the apparent absence of crystals. Some tubules were filled with neutrophilic leucocytes and eosinophilic casts. Degenerative changes were noted in other convoluted tubules.

To isolate and identify the crystalline material, portions of the kidneys were ground with a meat chopper and digested overnight at 37°C in artificial gastric juice. After straining and sedimenting, the material was washed with distilled water and centrifuged several times until the supernatant was clear. Microscopic examination of the sediment showed that considerable fine tissue debris was present. Therefore, the crystalline ma-

terial was suspended briefly in normal NaOH solution, centrifuged and repeatedly washed with several changes of distilled water. The crystals were assayed for DNC but a negative test was obtained.

The crystalline material was gray in color, insoluble in water, dilute acid and the common organic solvents. Likewise in dilute alkali the solubility was essentially nil. It burned somewhat sluggishly and left a slight residue which exhibited a typical brick-red color characteristic of calcium salts. The crude crystals darkened at temperatures above 300°; a slight residue was left unmelted at 350°. The melting point and solubility data suggested that the dinitrocarbanilide rather than the hydroxy dimethylpyrimidine portion of nicarbazin was the precursor of the isolated material. To obtain purified crystals of the metabolite, a 20 mg sample was dissolved in 2 ml of hot dimethylformamide, centrifuged to separate from foreign material, and then carefully diluted with 1 ml of water. After chilling and collecting the solid, the recrystallization was repeated once more. A third crystallization was then made from about 1 ml of dimethylformamide (without subsequent dilution with water). At this point the material was colorless, darkened above 335°C, and melted with decomposition >345°C. After drying at 100°C for 1 min. the material was subjected to elemental analysis and infra-red study. Qualitative tests for S and halogen were negative while a positive test for N was obtained. Found: C, 62.51, H, 5.45; N, 17.20. The infra-red spectrum (Nujol) contained bands, at 3.0-3.03, 6.0, 6.05, 6.32, 6.48, and 6.57 u. These bands are consistent with NH, amide carbonyl and aromatic residues. A check of the literature indicated that the properties of the isolated compound were in agreement with the previously described 4, 4′-bisacetamidocarbanilide(6). Analysis Calc. $C_{17}H_{18}O_3N_4$ C_1 62.5; H_1 5.56, N_1 17.17.

An authentic sample of the acetamidocarbanilide was prepared by fusion of p-acetamidoaniline and urea(6). No depression of melting point was observed for the mixture of the authentic sample and the isolated metabolite. Also the infra-red spectra of the 2 compounds were identical.

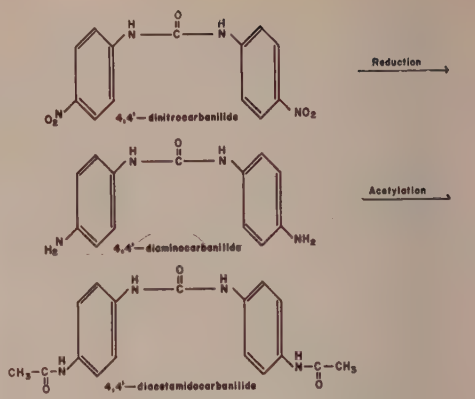


FIG. 2. Metabolic degradation of 4,4′-dinitrocarbanilide in the bovine.

To duplicate the proposed metabolic pathway (Fig. 2), a sample of 4,4′-dinitrocarbanilide was reduced catalytically to the diamine and subsequently acetylated via the Schotten-Baumann procedure. This preparation likewise yielded a compound identical with the metabolite from the kidneys of the calf.

Discussion. In calves, excessively large doses of nicarbazin produced kidney damage and crystalline deposits in the renal tubules. The material has been identified as 4,4′-bisacetamidocarbanilide and it is postulated that the compound was formed by reduction of the aromatic nitro groups of the dinitrocarbanilide component of nicarbazin. The newly formed amino groups were then acetylated into an insoluble metabolite. The pathological lesion and metabolic acetylation of this compound is similar to that resulting from administration of certain sulfonamides (7).

Other studies* have shown that large doses of nicarbazin produced kidney damage associated with crystal deposits in rats but not in dogs. It is well-known that rats possess a marked acetylating capacity whereas dogs are poor in this respect. Therefore, these *in vivo* observations support the suggestion that the metabolic pathway in the formation of 4, 4′-

* Unpublished data.

bisacetamidocarbanilide involves a reduction of DNC to the soluble diaminocarbanilide, which is readily excreted by dogs but is converted to an insoluble acetylated derivative in rats and calves. The *in vitro* demonstration of the reduction and acetylation of DNC to a compound identical with that found in the kidney tubules further supports the probability of this method for the metabolic degradation of nicarbazin in the bovine.

Summary. The feeding of large doses of nicarbazin to calves produced moderate nephrotoxicity associated with precipitation of crystalline deposits in the renal tubules. These crystals were identified as 4, 4′-bisacetamidocarbanilide. Additional evidence is presented that, after absorption, nicarbazin splits into its components, dinitrocarbanilide and hydroxydimethylpyrimidine, which are metabolized separately. The dinitrocarbanilide is first reduced to soluble diaminocarbanilide and then acetylated to insoluble 4,4′-bisacetamidocarbanilide. The renal tissue reaction and the metabolic degradation of 4,4′-dinitrocarbanilide resembles that known for the sulfonamides.

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Demonstration of Central Nervous Mediation of Acute Pulmonary Edema Produced by Intravenously Administered Epinephrine. (22722)

B. CASSEN, W. GUTFREUND, AND M. MOODY (Introduced by R. A. Monroe)

*Atomic Energy Project, School of Medicine, University of California at Los Angeles**

Experiments have been previously reported (1) on the phenomena associated with production of acute pulmonary edema in mice and rats. The most straightforward interpretation of the observations was obtained on the assumption that the genesis of the acute pulmonary edema either produced by air blast or by massive doses of epinephrine was mediated via the central nervous system. As other interpretations were not entirely ruled out, it was considered important to obtain a more rigorous proof of the central nervous mediation, especially when produced by epinephrine. Usually the physiological effects of intravenously administered epinephrine are interpreted in terms of its well-known peripheral effects. However, it can be easily demonstrated that massive and even lethal doses of epinephrine can be administered intravenously to cats without any evidence of acute pulmonary edema production, while the typical acute edema can often be produced by injection of epinephrine into the hypothalamic regions of the brain of the cat. A natural interpretation of these observations is that the cat has a blood-brain barrier more impervious to epinephrine than that of rodents.

To prove conclusively the central nervous mediation of the acute edema in rats, the experiment was performed of severing the spinal cords of a group of rats at a position between the second and third vertebrae. The respiration was maintained with a mechanical respir-

* This paper is based on work performed under contract No. AT-04-1GEN-12 between Atomic Energy Commission and the University of California at Los Angeles.

TABLE I. Spinal Severance.

Total body weights from 288 to 452 g (avg 351 g)

Lung wt in g	
1.923	
1.227	
2.295	(respirator blocked)
1.781	
2.570	(respirator blocked)
1.421	
2.665	(some jerking of hind legs)
1.705	
1.312	
1.417	
1.166	
2.683	(some jerking of hind legs)
1.665	
2.166	(some jerking of hind legs)
1.470	
Avg 1.831	

No Spinal Severance (But on Respirator).

Total body weights from 331 to 435 g (avg 383 g)

4.384	(nasal frothing)
8.550	
4.219	
3.610	(nasal frothing)
7.063	(*Idem*)
4.896	(")
Avg 5.453	

ator. Massive doses of epinephrine, that in controls produced tremendously edematous lungs, were administered intravenously. Under these conditions it would be expected that if the mediation was central nervous, no acute edema would occur in the cord-severed animals, while if the mediation was peripheral, the edema would develop.

Procedure. Rats were anesthetized as required with 0.4 ml to 1.0 ml of a one-gram/10 ml solution of Evipal Sodium administered intraperitoneally. They were laid on their backs and tied to a board by their feet. A tracheal incision was made and a cannula connected with a respirator was inserted and fastened, following well-established methods. The rats were turned over and refastened to the board. An incision was made in the back of the neck and a drill used to sever the cord between the second and third vertebrae. The animal showed a typical reaction to the severance by becoming momentarily rigid and then limp. Rats which withdrew their limbs on pinching were not considered to have their spinal cords completely severed and were not used. The rats were again turned over on their backs and freshly prepared adrenalin, 2 mg/kg, was injected into their femoral veins.

Results. Table I gives the measured lung weights of the experimental animals and a group of controls kept on the respirator and administered the same dose level of adrenalin, but without cord severance. However, incisions were made on the back of the neck of the controls, similar to those on the experimental animals.

These results strikingly demonstrate without the necessity of a statistical analysis that cord severance prevents development of acute edema. The appearance of the lungs in the spinal severance group was not normal. Some had a peculiar spongy appearance but no edema fluid was apparent. The production of simultaneous peripheral effects by the epinephrine is in no way excluded as a possibility. Also, possible mechanical irritation could arise from abnormal inflation during the artificial respiration.

Discussion. Luisada(2) has claimed that pulmonary edema can be mediated via the central nervous system by controlling capillary permeability of the pulmonary capillaries. As there is no anatomical evidence of nervous control of capillary permeability, this hypothesis is not well supported. A hypothesis for a different mechanism of central nervous mediation has been presented by us(1) which seems to fit in well with all the observations to date. In this mechanism only the central nervous control of precapillary sphincters is assumed.

An animal with severed cord on a respirator can be killed with a massive dose of epinephrine, even though no pulmonary edema is generated. It is likely but unproved that the epinephrine produces many other central nervous misfunctions besides that presumably produced on the circulating lung resistance by loss of control of the pulmonary precapillary sphincter system.

Summary. Fifteen rats with severed spinal cords, kept alive on a respirator, were given massive doses of epinephrine, that on controls produced consistently an intense acute pul-

monary edema. None of these rats developed the usual acute edema, and in 10 cases the lungs had normal weight. This result is interpreted as a proof of the central nervous mediation of the acute pulmonary edema produced by administration of epinephrine.

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Passive Transfer with Circulating Leukocytes of Delayed Hypersensitivity to Cat Scratch Antigen.* (22723)

WARREN J. WARWICK,† ARTHUR PAGE, AND ROBERT A. GOOD‡

Pediatric Research Laboratories of Variety Club Heart Hospital, University of Minnesota, Minneapolis

The passive transfer of delayed type skin hypersensitivity in animals and in man has been demonstrated towards simple chemical compounds(1), to tuberculin(2), to whole streptococcal cells, to SK-SD, and to streptococcal M substance(3,4). Such transfer has been possible with the leukocytes from peritoneal exudates, from lymph nodes, from the spleen, and from the thoracic duct, and from certain fractions of disrupted leukocytes(5,6). Hitherto the passive transfer of skin hypersensitivity to virus diseases has not been reported. It is the purpose of this paper to report the successful passive transfer of the delayed type skin hypersensitivity to cat scratch disease by means of circulating leukocytes. Cat scratch disease is a benign, subacute, regional lymphadenitis that is characterized by reticuloendothelial hyperplasia with epithelioid cell granulomas having central necrosis. Patients with cat scratch disease develop a characteristic skin reaction to an antigen prepared from pus aspirated from other cases of cat scratch disease. In its temporal relationships and its morphological features the reaction to cat scratch antigen has features similar to the tuberculin reaction and is characteristic of delayed allergy in man. No causative agent has been isolated from the suppurating lymph nodes from patients with cat scratch disease despite cultures on all commonly used media, upon inoculation into laboratory animals including rabbits, cats, monkeys, guinea pigs, mice, rats, and chick embryos, or on inoculation of tissue culture of HeLa cells, kidney cells, liver cells, or corneal cells.§ No microorganisms are to be seen in sections of the involved lymph nodes although cytoplasmic basophilic inclusion bodies have been described in the cells of such nodes(7). Further evidence in support of a viral etiology for cat scratch disease is that patients with this disease have a distinctly higher incidence of low complement fixation titers against the lymphogranuloma-psittacosis group viruses than does the population at large(8).

Materials and methods. Obtaining white blood cells. The antecubital area is cleansed with tincture of iodine, alcohol and green soap. The skin is infiltrated with 1% procaine hydrochloride. The antecubital vein is punctured with a 15 gauge needle and the blood is withdrawn through silicone-coated tubing into a silicone coated vacuum bottle containing ACD as anticoagulant. (For volumes of blood less than 400 ml heparin, 5 mg/100 ml blood, is used as the anticoagulant). Twenty-five ml of 4.7% dextran is

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† Alpha Phi Fellow in Cardiovascular Research.

‡ American Legion Memorial Heart Research Professor of Pediatrics.

§ Unpublished observations.

TABLE I.

Donor sensitive to cat scratch disease antigen			Recipient not sensitive to cat scratch disease antigen			No. of viable cells transferred (millions)	Mode of inoculation	Height of reactivity	Duration of reactivity, wk
	Sex	Age		Sex	Age				
C. D.	♀	11	M. M.	♀	3	260	Intramusc. + subcut.	2+	3
P. D.	♀	8	S. M.	♂	5	400	Intramusc. + subcut.	3+	>4
M. D.	♀	46	G. A.	♂	8	1000	Intramusc.	+	2

added for each 100 ml of blood, and the mixture inverted twice. (If heparin is used, normal saline is added in addition to the dextran, 29.5 ml/100 ml of blood). The mixed blood is allowed to stand at room temperature for 50 minutes, while red cells settle at a rate far in excess of the rate for the white cells. The plasma containing the white blood cells is removed with a plasma separating needle and a silicone-coated vacuum flask. The plasma is placed in silicone-coated centrifuge tubes and centrifuged at 1,000 rpm for 20 minutes. The supernatant plasma is poured off and the leukocytes together with about an equal number of admixed red cells are suspended in normal saline. The suspension is taken up into a silicone-coated syringe and injected intramuscularly, or partly intramuscularly and partly subcutaneously. *Handling the white blood cells.* An aliquot of the white cell suspension is used to determine the total and differential counts, and the percentage of viable cells is estimated by vital staining with trypan blue(9). Approximately 200,000,000 white blood cells are obtained/each 100 ml of blood processed, and between 80 and 99% are viable. The cell donors were patients convalescent from cat scratch disease who demonstrated a strongly positive skin test to cat scratch disease antigen. The recipients were children who had negative skin tests for cat scratch disease. Matching for compatibility of red cell groups was carried out because of the admixture of erythrocytes with the leukocytes. The cat scratch antigen was prepared from sterile pus aspirated from 2 carefully characterized cases of cat scratch disease. The pus was diluted 1:5 with pyrogen free normal saline and heated at 56 degrees C for one hour on 2 consecutive days. The resultant antigen in 0.1 ml quantities gave reactions characterized by induration and erythema beginning at 12 hours and lasting for 72 hours to 5-6 days after intradermal injection in subjects with typical cat scratch disease. Reactions on controls were completely negative. In the experimental series, skin tests were applied two days after the transfer of cells, and at weekly intervals thereafter. These tests were read for induration and erythema at 48 hours, as follows: +, 5 to 10 mm; 2+, 10 to 15 mm; 3+, 15 to 20 mm. Ancillary evidence of hypersensitivity was shown by the occurrence of a brownish discoloration after the erythema had faded. This discoloration, which persisted for many weeks in patients with cat scratch disease, lasted as long as the passively transferred reactivity could be demonstrated.

Results. The results summarized in Table I demonstrate the successful passive transfer of cat scratch disease skin hypersensitivity by means of circulating leukocytes in three consecutive cases.

An attempt to transfer the skin reactivity to cat scratch disease by means of plasma from a convalescent patient with a strongly positive skin reaction (4+) was unsuccessful. Four hundred cc of blood were removed from patient M.D. into a standard blood collecting flask with ACD as anticoagulant. After settling of cells the plasma was removed and filtered through a Seitz filter. One hundred and fifty cc of this plasma were given intravenously to recipient M.M. This patient failed to develop positive skin reactivity to cat scratch disease antigen.

Summary. Delayed type hypersensitivity to cat scratch disease can be passively transferred by blood leukocytes, but not by plasma from hypersensitive donors convalescent from

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Effects of Azetazoleamide (Diamox) Upon Blood Sugar of Normal and Diabetic Patients. (22724)

ARTHUR J. GROSSMAN AND ROBERT C. BATTERMAN

Department of Medicine, New York Medical College, Metropolitan Medical Center and Bird S. Coler Memorial Hospital and Home for the Aged, New York City

Moseley *et al.*(1) observed insulin shock in a diabetic patient receiving azetazoleamide as a diuretic and subsequently made studies on normal subjects to evaluate the effect of this drug on glucose tolerance. No significant change in the intravenous glucose tolerance curve was noted when studied with and without the administration of azetazoleamide. They concluded from the oral glucose tolerance curve that azetazoleamide may interfere with the absorption of glucose from the gastrointestinal tract.

Since Moseley did not study diabetic patients and limited his study to an acute problem, our studies were designed to determine whether azetazoleamide influences the fasting blood sugar of normal and diabetic patients after acute and chronic administration and whether the oral glucose tolerance curves can be affected.

Acute study. Acute experiments were carried out in 20 normal and 20 diabetic subjects. Half of each group were given either 500 mg of azetazoleamide* orally and fasting blood sugars determined (method of Folin-Wu)(2) 3 hours later or followed without drug administration for a similar period. One week later, to eliminate biologic variation, the subjects were re-studied but the procedure reversed. The results of this experiment are summarized in Table I. Azetazoleamide did not alter the fasting blood sugar 3 hours after its administration in the normal and the diabetic group of patients when studied as an acute experiment. The blood sugar taken at 3 hour intervals was practically the same with and without the drug.

Chronic study. Chronic experiments were carried out in 6 normal and 6 diabetic patients. Azetazoleamide was given in doses of 500 mg daily for a 3 week period to 3 normal and 3 diabetic patients. Fasting blood sugars were determined (method of Somogyi)(3) at weekly intervals. As a control, a similar group was followed without drug administration. In Table II average values of blood sugars are recorded. With chronic administration of 500 mg of azetazoleamide there was no significant change in the fasting blood sugar in either the control patients or those receiving the drug for a period of 3 weeks.

Oral glucose tolerance. The final experiment was related to the effect of azetazoleamide on the glucose tolerance curve. Three normal and 3 diabetic patients received the standard oral glucose tolerance test utilizing 100 g of glucose. The procedure was repeated at a later date with 500 mg of azetazoleamide being given 3 hours prior to the

* Supplied by Lederle Laboratories Division, American Cyanamid Co.

TABLE I. Influence of Azetazoleamide upon Blood Sugar of Normal and Diabetic Patients. (Value of fasting blood sugar in mg %.)

	Without drug				After oral admin. of 500 mg			
	Control		3 hr		Control		3 hr	
Subjects	Range	Avg	Range	Avg	Range	Avg	Range	Avg
Non-diabetic (20)	95-132	114	100-124	112	83-140	112	93-132	113
Diabetic (20)	85-380	233	83-320	202	106-367	237	103-340	222

TABLE II. Chronic Experiment on Normal and Diabetic Patients. (Avg value of fasting blood sugar in mg %.)

	Control*				500 mg azetazoleamide O.D. daily*			
Weeks	0	1	2	3	0	1	2	3
Non-diabetic	86	84	91	97	79	76	82	87
Diabetic	137	135	144	158	118	118	120	127

* 3 subjects for each group.

administration of glucose. The glucose tolerance curves are recorded in Table III. Although there was considerable individual variation in the curves obtained, the blood sugar curve for each of the subjects studied was not different with and without azetazoleamide. The differences in the curves obtained are within the range of technical and biologic variation.

Comment. Maren *et al.*(4) previously observed in chronic acidotic dogs maintained on azetazoleamide that the fasting blood sugar had slight but definite tendency to rise. However, an insulin tolerance test was found to be normal in a dog treated with azetazoleamide.

The response that Moseley noted was probably due to a well recognized clinical observation of diabetic patients with congestive heart failure. During this time they may be uncontrollable and require an increased dose of insulin. As the congestive heart failure improves, the insulin requirements may be decreased and eventually discontinued. Since azetazoleamide is used in diabetic patients for its diuretic properties, it is conceivable that the drug resulted in a change of cardiac status and insulin requirements, and azetazoleamide *per se* has no significant effect on sugar metabolism or insulin sensitivity. Any diuretic agent would have the same action and it is unnecessary to postulate a specific effect of azetazoleamide upon the blood sugar of diabetic subjects.

Conclusions. 1. The influence of azetazoleamide upon fasting blood sugar and the glucose tolerance curve was studied in a group of diabetic and normal subjects. Azetazoleamide was given either as a single dose or for a period of 3 weeks. 2. No significant alteration of the fasting blood sugar or the glucose tolerance curve was noted following the administration of azetazoleamide.

TABLE III. Effect of Azetazoleamide on the Glucose Tolerance Curve. (100 g glucose orally —value of fasting blood sugar in mg %.)

		Control				500 mg azetazoleamide 3 hr before test			
	Min.	0	30	90	150	0	30	90	150
Non-diabetics	1. A.B.	76	109	107	100	79	106	106	101
	2. C.P.	88	138	126	115	90	123	104	101
	3. J.K.	89	162	200	138	100	151	132	112
Diabetics	1. H.D.	127	200	308	313	128	181	268	258
	2. A.F.	96	99	172	236	107	112	189	245
	3. H.B.	159	192	267	300	178	213	251	305

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Failure to Demonstrate Capsular Swelling in *Cryptococcus neoformans.** (22725)

E. Edward Evans, H. P. R. Seeliger,† Lottie Kornfeld,‡ and Corina Garcia§

Department of Microbiology, University of Alabama Medical Center, Birmingham, and Dept. of Bacteriology, University of Michigan, Ann Arbor

The capsule which surrounds certain microorganisms is ordinarily water-clear and difficult to see without appropriate staining procedures. If, however, specific antibody is added, a reaction occurs which renders the capsule clearly visible. This antigen-antibody reaction which was first demonstrated with the pneumococcus(1) has been of interest not only for identifying and classifying microorganisms, but also because it affords direct cytological evidence for the combination of antibody with a surface antigen. Although this combination is frequently referred to as the "Quellung" or "capsular swelling" reaction, several investigators(2-5) have recently suggested that the capsule does not actually increase in size in bacteria like pneumococcus, the *Klebsiella* group and the *Bacillus* group. Tomcsik in discussing the morphological arrangement of immunopolysaccharides in the bacterial cell(5) suggested that the essential feature of the reaction is precipitation and that swelling might be a secondary phenomenon due probably to capsular hydration. Johnson and Dennison(6) presented experimental data which indicated that a volume increase actually occurs when the pneumococcus capsule reacts with antibody. This is in conflict with the results of the previously mentioned authors as well as with work on the capsular reaction of *Cryptococcus neoformans* by Neill, *et al.*(7) and by members of our group(8).

The purpose of the present study is to describe further experiments on this interesting reaction. *Cryptococcus neoformans* has been used as the test organism since its relatively large size and spherical shape facilitate direct microscopic measurements.

Methods. Two strains of *C. neoformans* were selected because of their ability to produce relatively large capsules. Both strain DU (Type A) and strain L2 (Type B) were isolated from patients at the Los Angeles County General Hospital. Cultures were maintained on neopeptone-glucose medium (8). In certain experiments, cells with larger capsules were obtained by injecting mice intraperitoneally with 4 x 10^9 *C. neoformans* and washing the cells from the peritoneal cavity with saline solution on the sixth day of infection. Phenol, 0.5%, was added to cell suspensions that were to be stored for a period of time. All cells were centrifuged and washed 4 times with saline solution immediately before use to remove dissolved polysaccharide. Antiserum was prepared in rabbits as described in an earlier report(9). Antiserum No. 12 was polyvalent with respect to Types A, B, and C. Serum taken from rabbits before immunization was used for control experiments. Among the methods used to study possible change in capsular size during the antigen-antibody reaction was the measurement of packed volumes of centrifuged cells with and without added antibody. For these experiments, strain DU was grown in neopeptone-glucose broth for 4 days at 25-27°C with continual agitation, and then killed by the addition of phenol to a final concentration of 1%. Suspensions of strain L2 from infected mice were also used. Cells were removed by centrifugation, washed four times with saline solution and resuspended in saline or phosphate buffer solutions at pH 7.0 ($\mu = 0.1$). The cell concentration was adjusted to 85% transmittance in a Klett Summerson

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† Permanent address: Hygiene-Institut, Universitat Bonn, Germany. Temporarily assigned to Department of Microbiology, University of Alabama Medical Center during tenure of a W. H. O. Fellowship.

‡ Present address: Department of Medicine, University of Chicago.

§ Present address: Avenida Vilaflor, Quinta Puerto Rico, Sabana Grande, Caracas, Venezuela.

TABLE I. Volumes of Packed Cells following Two Hours Centrifugation at 2500 × Gravity.

Diluent	Cell strain	Serum dilution	Anticryptococcal serum #12 (mm^3)	Normal rabbit serum (mm^3)
Phosphate buffer pH 7 ($\mu = 0.1$)	DU	1: 4	12	13
		1: 16	12	13
Saline solution	"	1: 4	13	14
		1: 16	13	13
Saline solution	L2	1: 8	30	37
		1:160	30	36

DU strain was harvested from cultures and the L2 strains from infected mice. Capsules of the latter strain were extremely large (see Table III for mean diameters).

Colorimeter using a No. 54 filter. Both normal rabbit serum and anticryptococcal serum for use in the experiment were subjected to preliminary centrifugation at 2500 x g for 30 minutes although both had been clarified by filtration. To Hopkins centrifuge tubes were added 4 ml of cell suspension and 4 ml of a given serum dilution. Following a 5 minute incubation at room temperature, a sample was taken for microscopic examination to verify the presence or absence of a capsular reaction. The tubes were then centrifuged at 2500 x g for 30 minutes and the cell volume noted. The tubes were centrifuged at the same force for additional 30 minute periods until a total time of 2 hours centrifugation was reached. Similar experiments were conducted at 400 x g and 1700 x g and with saline or cultural supernate replacing the phosphate buffer as diluent. Although other investigators have found forces above 2500 x g necessary for demonstrating volume changes in pneumococcus(6), increasing the force above this level did not alter the relative volume in experiments with cryptococcus. For direct microscopic measurement of capsular diameter, suspensions containing 10^7 cells per ml of saline solution were used. In one procedure, individual cells were located on a slide containing no serum and just enough India ink to permit the capsular outline to be seen. The diameters of the capsule and of the somatic portion of the cell were then measured with a Carl Zeiss filar micrometer. Following this, diluted antiserum was allowed to flow under the cover glass while the cell was held in the microscopic field. The same cell was measured again after it had produced a capsular reaction. In other experiments, 0.2 ml portions of cell suspension were placed in 10 x 75 mm test tubes. To some tubes were added 0.04 ml of antibody solution, to others, 0.04 ml of dilute India ink. After mixing, a drop of each was added to cover glasses for the preparation of hanging drop or flat slides. In other experiments, a counting chamber of uniform (0.1 mm) depth was used. In some instances, India ink was added to slides containing cells plus anticryptococcal serum and to control slides with cells and normal serum. After an incubation period of 5 to 15 minutes at room temperature, the diameters of 30 cells were measured with an ocular micrometer. The averages of these values were then calculated.

Results. The results of centrifuging Cryptococcus (DU) cells for 2 hours at 2500 x g are presented in Table I. There was no significant difference in packed volumes of aliquots of cells which had been mixed with specific antiserum and with normal rabbit serum. The substitution of saline for phosphate buffer had no effect on the results. Decreasing the force to 1700 or 400 x g failed to disclose any volume increase in the cells mixed with antiserum. In some experiments, the volume was slightly greater in normal serum which could have been due to shrinkage or to firmer packing in the antigen-antibody aggregate. When the experiment was repeated with cells possessing extremely large capsules (diameter 28 to 50 μ), there was evidence of capsular shrinkage in the presence of antibody. Dilution of the antibody to the point where the capsular reaction was relatively weak (one twofold dilution be-

TABLE II. Effect of Dissolved Polysaccharide in Cultural Supernate on Apparent Packed Cell Volume.

Diluent added to washed cells	Serum (1:8) Anticryptococcal #12 (mm^3)	Serum (1:8) Normal rabbit serum (mm^3)
Saline	10	10
Cultural supernate		
1: 4	>50*	11
1:20	39	10

* The packed cell-precipitate mass greatly exceeded the graduations in the tube. The vol was estimated to lie between 300-400 mm^3.

Tubes were centrifuged at 2500 × gravity for 2 hr.

Strain DU was used in this exp.

low the extinction point) did not change the relative difference between tubes with antiserum and those with normal serum.

The effect of extracapsular polysaccharide on the apparent volume of packed cells is shown in Table II. It may be seen that when washed cells were resuspended in the culture fluid in which they had been grown and then mixed with antiserum, a great increase in volume resulted as compared to values in normal serum. This represents precipitation of dissolved polysaccharide and possibly other antigens present in the supernatant cultural fluid.

Direct microscopic measurements on single cells before and after the addition of antibody demonstrated that there was no swelling and indeed, the capsules appeared to shrink when antibody was allowed to flow under the cover glass. Control slides in which additional India ink was substituted for antibody also showed some degree of shrinkage, indicating that at least part of the observed decrease in diameter was due to mechanical lifting of the cover glass, permitting the flattened capsule to assume a spherical shape. Because of the technical shortcomings of this method, another procedure was adopted. Two hanging drop slides were prepared, one with cell suspension plus antiserum, the other with cell suspension plus India ink. The diameters of 30 cells were measured on each slide, and the mean diameter calculated. Similar experiments were set up using a counting chamber of uniform depth and a flat microscope slide with a cover glass. In no case was capsular swelling observed (Table III); moreover, mean capsular diameters of the cells in antiserum were significantly lower than those in India ink.

Discussion. In studying the mechanism of this antigen-antibody reaction, it is important to know whether swelling of the capsular polysaccharide-antibody complex is an essential feature of the reaction. The data presented here indicate that it is not. The difference in our results and those of others(6) cannot be adequately explained without further work. A different organism, the pneumococcus, was used in the earlier study and it is possible that the capsular reaction differs among different species or types depending on the physicochemical nature of the capsular antigen.

The pneumococcus is known to have an extra-capsular slime layer(2,10) and this may be responsible for the apparent swelling in the pneumococcal capsule. It may be significant that previous investigators(6) did not wash their cells to free them of extra-capsular polysaccharide. In their centrifugation experiments, the apparent increase in volume may have been due to precipitate. In our experiments with cryptococcus, a 1:4 dilution of cultural supernate added to washed crypto-

TABLE III. Comparison of Means of Capsular Diameters in India Ink and in Antiserum.

Exp. No.	Treatment	Mean diameter (μ)	S.D.	S.E.	P value
1	India ink	36.72	2.18	.40	<0.001
	Antiserum	34.62	2.00	.37	
2	India ink	38.43	3.22	.59	"
	Antiserum	34.90	2.66	.49	
3	India ink	38.00	3.43	.63	"
	Antiserum	34.76	2.49	.46	
4	India ink	43.86	2.28	.42	"
	Antiserum	41.16	2.21	.40	

Means were calculated from measurements on 30 cells each. Exp. 1 was done with a hanging drop slide; Exp. 2 and 3 in a counting chamber; Exp. 4 on a flat slide with a cover glass.

Abbreviations: S.D., stand. dev.; S.E., stand. dev. of the mean; P, probability calculated by Student's "t" test.

Strain L2 harvested from mice was used for these experiments.

coccal cells yielded so much precipitate that the total mass remained far above the graduated level in Hopkins tubes. The difference between this and the normal serum control was indeed striking, and could have been interpreted as capsular swelling had not the experiment with washed cells been conducted.

Although there is no capsular swelling, the data presented here suggest that there is some degree of capsular shrinkage. This is particularly apparent in cells harvested from mice because these cells have extremely large capsules. Further experimentation will be necessary to determine whether this apparent shrinkage has biological significance although from a statistical standpoint the differences in mean diameters presented in Table III are significant. It is possible that the apparent shrinkage seen under the microscope does not represent any volume change in the capsule but merely the change from an ellipsoid to a sphere during the antigen-antibody reaction. A shortcoming of the direct microscopic measurement is the fact that capsular boundaries in India ink are not as sharp as in antiserum, particularly in the counting chamber where the fluid depth is 0.1 mm. This accounts for the somewhat larger standard deviations obtained in counting chamber experiments. This criticism cannot be directed at centrifugation experiments; however, it is possible that the shrinkage observed here represents better packing in the presence of antibody.

Summary. 1. In studying the mechanism of the "capsular reaction" one of the first questions to be answered is whether or not the capsule swells when antibody is added. Data presented here indicate that it does not, using *C. neoformans* as a test organism. Exposure of washed, encapsulated *C. neoformans* to antibody results in a prominent clarification of capsular outline, but no increase in capsular size as determined by packed cell volume after centrifugation and by direct microscopic measurement. When cells with large capsules are employed, there is an apparent *decrease* in capsular size during the reaction with antibody. 2. If capsulated cells are not washed free of soluble capsular polysaccharide present in the cultural supernate, the soluble antigen coprecipitates when antibody is added. This may lead to the erroneous conclusion that there is an increase in capsular volume.

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Effect of Biotin Deficiency on Follicular Melanocytes of Mice. (22726)

WALTER C. QUEVEDO, JR.* (Introduced by H. M. Patt)

Biology Department, Brown University, Providence, R. I.

It has been known for some time that pigmented mice and rats produce grey hairs when made biotin deficient(1,2,3). Unlike greying induced by X-rays, which is permanent(4), greying resulting from biotin deficiency is reversible(3). It would appear that melanocytes persist within the hair follicles of biotin deficient mice whereas they are destroyed in X-irradiated mice. Thus far,

* Predoctoral Fellow of the U. S. Public Health Service. Present address: Argonne National Laboratory, Lemont, Ill.

no attempt has been made either to trace the fate of melanocytes within the greyed hair follicles of biotin deficient mice or to determine the extent to which such depigmented melanocytes are morphologically and physiologically similar to the "pigmentless" melanocytes (clear cells) of albino mice. In addition, whereas it is well established in mice that the synthesis of the black-brown melanins (eumelanins) is inhibited by biotin deficiency, it is not known whether yellow melanin (phaeomelanin) synthesis is similarly affected.

It is the purpose of this paper to describe the effects of biotin deficiency on the follicular melanocytes of both yellow (A^y) and black (C57BL) mice.

Materials and methods. To produce biotin deficiency, 25 black (C57BL) and 5 yellow mice, 16 days of age, were weaned to diet #9A described by Wilson *et al.*(2). The effectiveness of this diet in causing greying of hair in C57Black mice was demonstrated by these investigators and subsequently by Rauch(3). Biopsies of skin were taken approximately 20 days post-weaning, shortly after the hairs of the second coat grew visibly above the skin. The skin samples were placed in Bouin's fixative and routine histological procedures were followed. Paraffin sections were cut at 7 μ and stained with Delafield's hematoxylin. Sections of depigmented follicles of the biotin deficient mice were compared with the follicles of albino mice in a comparable stage of hair growth. The activity of the dopa oxidase enzyme system within the depigmented melanocytes was determined by testing their response to exogenously supplied d,l-3-4-dihydroxyphenylalanine (dopa). Fresh frozen sections of skin from biotin deficient C57Black mice were cut on the freezing microtome at 30 μ and incubated in a buffered 0.76M solution of dopa according to the method of Laidlaw and Blackberg(5). Control sections were incubated in buffer alone. In addition, fresh frozen sections of skin from normal C57Black mice were treated in identical fashion, some sections being incubated in dopa solution, others in buffer alone.

Results. The pattern of greying is the same in C57Black mice and in yellow mice weaned to the experimental diet. The first hair growth cycle is completed approximately 2 days post-weaning when the mice are 18-19 days old. The hairs of the first generation are normal in pigmentation. The hairs of the second generation, appearing above the skin at approximately 20 days post-weaning, show a marked reduction in pigment content. Longitudinal sections of the hair follicles reveal a similar reduction in the amount of pigment within the hair bulbs. Except for the presence of small granules of pigment within the melanocytes and recipient epithelial cells, the greyed hair follicles are strikingly similar to those of the albino (Figs. 1, 2). The depigmented melanocytes contain a large hyalinated cytoplasm as do the clear cells or "pigmentless" melanocytes of the albino. Depigmented melanocytes are found in the hair bulbs of both biotin deficient black and yellow mice and in all instances they are restricted to the upper halves of the hair bulbs. This is also the position of clear cells within the hair bulbs of the albino mouse.

Depigmented melanocytes resulting from biotin deficiency react positively to exogenously supplied dopa. Abundant dopa melanin is observed in the hair follicles which are incubated in dopa solution (Fig. 3). The amount of dopa melanin formed indicates clearly that with regard to the activity of the dopa oxidase system, the melanocytes are capable of greater melanin synthesis than is expressed in the biotin deficient individual. In this way depigmented melanocytes differ from clear cells which have been shown to be dopa negative(6).

Discussion. The present experiments demonstrate that melanocytes persist within the depigmented hair follicles of biotin deficient pigmented mice and that they are similar in morphology to the follicular clear cells of albino mice. This observation lends support to the view held by previous investigators (7,8) that the clear cells of the albino are melanocytes which are incapable of melanin synthesis due to a gene controlled enzymatic deficiency. Unlike clear cells, however, de-

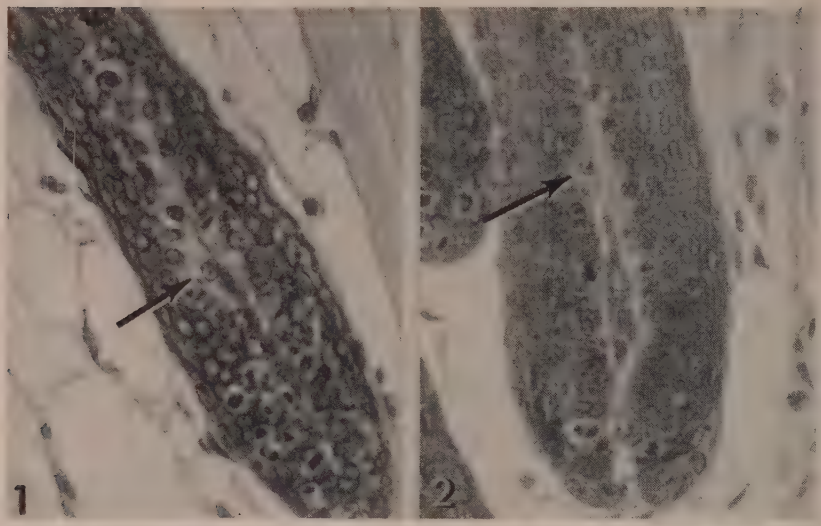


FIG. 1. Vertical section through a hair follicle of the albino mouse. Note the large clear cells (arrow) in the upper bulb. ×430.

FIG. 2. Longitudinal section of a hair follicle from a biotin deficient C57 Black mouse. Depigmented melanocytes (arrow) are visible in the upper bulb. ×430.

pigmented melanocytes retain the ability to respond *in vitro* to dopa. It is probable that the histological appearance of greyed hair follicles observed in biotin deficiency is also typical for the reversibly greyed hair follicles resulting from other nutritional deficiencies, *e.g.*, copper, zinc, and pantothenic acid deficiency(9). However, it would not be surprising if the response of depigmented melanocytes to exogenous dopa is subsequently found to vary considerably with the type of nutritional deficiency.

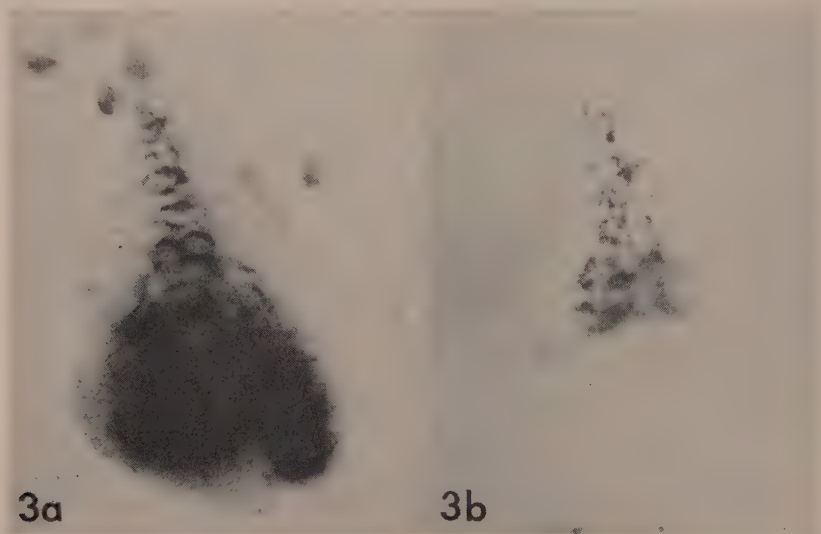


FIG. 3. a. Frozen section of a hair follicle from a biotin deficient C57 Black mouse, following incubation of the section in dopa. ×430. b. Control section to that shown in a., incubated in phosphate buffer only. ×430.

It would appear that both phaeomelanin and eumelanin synthesis are equally sensitive to biotin deficiency. In both yellow mice and black mice deficient in biotin a marked reduction in pigmentation is found in the hairs of the second hair generation. At present, with the role of biotin in the pigmentary process undetermined, no statement can be made as to the nature of this inhibition.

Summary. Both yellow (A^y) and black (C57BL) mice produce grey hairs when weaned to a diet known to cause biotin deficiency. Histologically, the greyed hair follicles of biotin deficient mice bear a striking resemblance to the hair follicles of albino mice. The depigmented melanocytes of biotin deficient mice are similar in morphology to the follicular clear cells of the albino. Unlike clear cells, however, depigmented melanocytes react positively to tests for the presence of dopa oxidase.

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Measurement of Total Body Water in Sheep Using I^{131} Labeled 4-Iodo-Antipyrine. (22727)

SAM L. HANSARD AND W. A. LYKE

University of Tennessee, Atomic Energy Commission, Agricultural Research Program, Oak Ridge, Tenn.

Antipyrine has been employed extensively as an indicator for the *in vivo* estimation of total body water in man(1,2) and animals (3-6). The method is based upon the dilution principle and is an adaptable and economical procedure for studies with the intact animals. Despite certain theoretical shortcomings(7,8) this method has been found to compare favorably with parallel procedures for body water measurement employing whole body analysis(3-5), specific gravity(6,9,10, 11) or the isotope dilution technic using deuterium(7), or tritium oxide(12). However, these methods require tedious procedures of sample preparation and time consuming analyses that discourage their use in routine experimentation. The recent introduction of I^{131}-labeled 4-iodo-antipyrine as a simple and rapid method for estimating total body water in man(13) made feasible the adaptation of this procedure for use with farm animals(14). This study is concerned with comparative behavior and volume of distribution of antipyrine and I^{131} labeled 4-iodoantipyrine for total body water estimation in sheep.

Materials and methods. The animals used in these studies were normal, healthy individual wether lambs and mature sheep maintained under typical summer and fall farm management conditions. The procedures employed for handling animals and performing quantitative injections of test substances have been described(15). Animals were taken off

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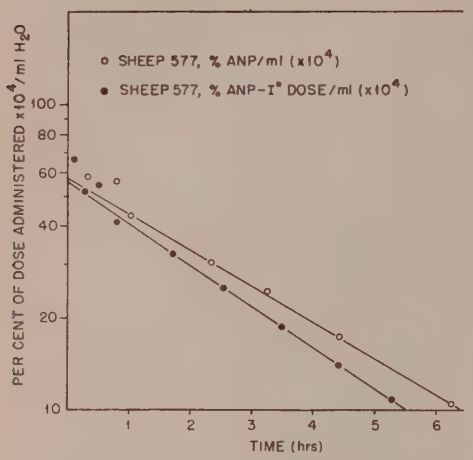


FIG. 1. Log concentration of antipyrine and I^{131} 4-iodo-antipyrine in blood of an avg sheep (577) regressed on time in hr after inj. by method of least squares to derive the theoretical concentration of total body water.

feed and water 12 to 15 hours prior to dosing intravenously with approximately 2 grams antipyrine and/or 50 microcuries I^{131} labeled 4-iodo-antipyrine/100 lb body weight. Venous blood and urine samples were taken periodically during the first 5 hours and at 24 hours after injection. The blood was centrifuged and plasma removed. Radioactivity was measured in four ml of plasma using a well-type sodium iodide scintillation detector attached to a conventional type scaler unit. Radioactivity in an aliquot of standard solution was measured in the same way for calculation of relative iodine content. Total antipyrine concentration was determined by precipitation procedure(16). Radioactivity in both plasma and zinc filtrate fraction was measured for comparative purposes, and for estimation of plasma protein binding. The volume of distribution for each test substance was then calculated by plotting concentration against time after injection on semi-logarithmic paper and exterpolating back to zero time by the method of least squares. (Fig. 1). The zero value was corrected for plasma water by dividing by 0.93, and the test substance injected divided by the concentration in plasma water to give total volume of body water. Body water in liters divided by body weight (kg) X 100 gave the % body water. Thyroid uptake of I^{131} was checked periodically and at 24 hours after administration of labeled 4-iodo-antipyrine using an adapted scintillation probe attached to a count-rate meter.

Results. The adaptation of a new test substance requires that certain physiological studies be made for comparison with proven and acceptable methods of analysis. Since the antipyrine procedure has been used generally for estimation of body water volume in man and animals and has compared favorably with various other procedures(4,5,11, 13), it was of interest to consider results from iodinated antipyrine with data obtained concurrently with stable antipyrine. The use of the labeled compound further permitted a simple investigation of rate and behavior patterns of test substances.

Partition in the plasma. The uniform distribution of both antipyrine and the I^{131} labeled 4-iodo-antipyrine in sheep occurs in about one hour (Fig. 1). The initial rate of disappearance for the 2 test substances was not much different, averaging approximately 30% per hour for animals in the 2 age groups. If it is assumed that the labeled and carrier antipyrine are similarly metabolized by sheep, then the degree of antipyrine binding by plasma proteins may be determined simply by measuring plasma radioactivity before and after deproteinization with zinc hydroxide (1). Furthermore, if plasma could be used directly without deproteinization to reflect the water content from radiochemical analysis, the procedure would be simplified. Therefore, all blood samples were centrifuged and aliquots measured for radioactivity before and after deproteinization and the % reversibly bound to plasma(1) proteins calculated

$$\text{as } 100 \times \frac{\%\text{ dose plasma } I^{131}/\text{cc minus } \%\text{ dose filtrate } I^{131}/\text{cc}}{\%\text{ plasma } I^{131}/\text{cc}}.$$

Results from analysis of plasma samples taken periodically from sixty-two sheep indicated that 10 to 20% of plasma iodine was bound to proteins during the critical 5 hour test period and increased to 60% of that remaining in the blood at 24 hours. In quantitative studies where *absolute* volume data are

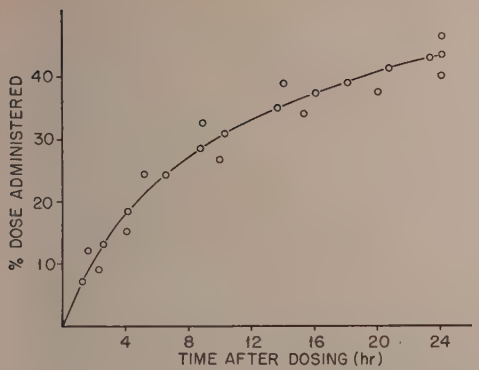


FIG. 2. Accumulative percent excretion of I^{131} in urine of sheep dosed intrav. with I^{131} labeled 4-iodo-antipyrine.

required corrections, therefore, would be necessary. However, in those studies where relative body water volume values are desired, the simplicity of the plasma analysis procedure would be justified.

Thyroid uptake. Measurements of thyroid uptake of I^{131} from labeled antipyrine were made periodically throughout the test period by external counting over the thyroid area with an adapted probe type scintillation counter. The fact that only 3 to 5% of the dose administered was found in this area during the 5 hour test period would indicate the degree of firmness of I^{131} binding to the antipyrine. Six wethers were sacrificed after 24 hours and the thyroids analyzed radiochemically were found to contain 35 $\pm$ 8 percent of the I^{131} administered.

Excretion of metabolized antipyrine. Urinary and fecal excretion values and radioanalysis of gastrointestinal tract contents from 8 sheep sacrificed 24 hours after administration of test substances indicated that insignificant amounts of I^{131} were excreted in feces. The accumulative average % of administered dose excreted in urine of 12 sheep is shown in Fig. 2. Of 44% voided during the first day, approximately 20% was excreted during the critical 5 hour test period of the study.

Total body water in sheep. The results of total body water determinations using both antipyrine and I^{131} labeled 4-iodo-antipyrine procedures with 62 young and mature sheep are shown in Table I. These values for young lambs are slightly higher than the 59 $\pm$ 5.1 and 61 $\pm$ 3.4% reported earlier(14) using antipyrine and I^{131} labeled 4-iodo-antipyrine concurrently in 36 trials with 12 growing lambs. This is probably due to the greater number of younger lambs used in the current study since the young animals have invariably demonstrated a higher body water volume. Mature wethers showed a lower body water volume and checked closely with reports for beef cattle(3), averaging 55.3 $\pm$ 6.3 and 56.2 $\pm$ 6.6% body water respectively by the antipyrine and labeled antipyrine procedure. The range shown for the two methods was not much different, and reflected the rate of disappearance from plasma (Fig. 1), indicating results from the isotope procedure to compare favorably with that for the stable antipyrine method. The advantage of the labeled antipyrine method lies in simplicity and possible rapidity. It is necessary only to inject intravenously and quantitatively the activity in a minimum volume and to measure the radioactivity in the plasma of blood samples drawn periodically over a 1 to 5 hour period (13). The fact that the labeled test substance appeared to give a slightly higher body water volume may well be accredited to inherent errors involved in quantitative admin-

TABLE I. Percent of Body Water in Young and Mature Sheep as Measured by Antipyrine and I^{131} Labeled 4-Iodo-Antipyrine.

Age	Wt, lb	No. of animals	Method*	Mean†	Stand. dev.	Range
Young	65 $\pm$ 10	34	A.N.P.	61.8	6.5	45-72
	62 $\pm$ 12	44	I^{131} A.N.P.	62.3	6.8	48-74
Mature	135 $\pm$ 49	16	A.N.P.	55.3	6.3	37-63
	138 $\pm$ 51	18	I^{131} A.N.P.	56.2	6.6	35-65

* Refers to antipyrine (A.N.P.) and I^{131} labeled 4-iodo-antipyrine (I^{131} A.N.P.).

† Percent body water calculated as $100 \times \frac{\text{Body water (l)}}{\text{Body wt (kg)}}$.

istration of very small volumes of solution. The activity usually required was contained in 0.25 to 1.0 ml. It should be noted, however, that for dilution methods in general the experimental errors almost invariably lead to high values for body water. Some of the more important sources of error include (1) nonquantitative injection into the blood stream, (2) excess water in the gastrointestinal tract and, (3) rapid metabolism of the test substance or its removal from the circulating blood following administration. In this study every precaution was taken to eliminate or to correct for these errors, and by management practices to avoid physiological changes that are known to affect the normal individual.

Discussion. The ultimate objective of establishing a predictable relationship between body water and weight in farm animals is obviously that of using such data to evaluate gain or loss of body energy in nutritional investigations or physiological studies without the need for sacrificing the animal. Several investigators have demonstrated the relative merits of estimating body fat from body water measurements(3,4,5,11,14) and mathematical expressions have been proposed that would permit estimations for other body constituents(17). It is evidenced from the data presented that, although the I^{131} labeled 4-iodo-antipyrine procedure does not rule out all of the recognized shortcomings of the antipyrine method, it offers a simple and rapid means of estimating *relative* body water in mature and in growing farm animals, and is well adapted for routine application to experiments requiring a measurement of this vital metabolic pool. These values employed, for the calculation of total body fat and their extension to prediction of chemical composition and energy values(17) would thereby provide additional criteria for the response measurement of animals to various experimental treatments.

Summary. A simple and rapid procedure is presented for the *in vivo* measurement of total body water in sheep using I^{131} 4-iodo-antipyrine as the test substance. Body water values for 62 young and mature sheep averaged 61.3 $\pm$ 6.5 and 55.3 $\pm$ 6.3, respectively, by the antipyrine method, and 62.3 $\pm$ 6.8 and 56.2 $\pm$ 6.6%, respectively by the labeled antipyrine procedure. Thyroid uptake of I^{131} from labeled test substance amounted to 3 to 5% and urinary excretion was less than 20% of administered dose during the critical 5-hour test period. Protein binding was evidenced, indicating that in sheep both test substances were reversibly bound to plasma protein. Consideration is given to the use of these body water volume values in establishing a predictable relationship to body constituents and to provide additional criteria for estimating animal response to experimental treatments.

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Characterization of an Antibacterial Peptide from Calf Thymus.* (22728)

Robert C. Skarnes and Dennis W. Watson

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis

Many investigators have succeeded in extracting antimicrobial substances from various normal tissues during the past several decades. Some of these factors have been shown to be basic proteins or peptides. Bloom *et al.*(1) obtained an anthracidal substance from calf thymus and other tissues. This material was found to be stable to acid and heat, of polypeptide nature, and basic due to the presence of a large amount of lysine. That this antibacterial peptide was derived from a histone has been suggested(16). A mycobactericidal thymus peptide has been described in a series of papers by Dubos and Hirsch (5,7,8). Upon chemical analysis it was shown to be basic, possessing large amounts of lysine and arginine, and having an isoelectric point of 10-11.

This investigation is concerned with further studies and characterization of the basic thymus peptide of Bloom and co-workers(1).

Methods and materials. The tissue peptide was prepared from fresh calf thymus according to the method of Bloom and Prigmore(3) with one modification. The final S-IV product was brought to half saturation with ammonium sulfate at room temperature and pH of 6.0. The supernatant solution was dialysed free of salt and then lyophilized and weighed. Stock solutions of the thymus peptide were made up in distilled water at a concentration of 0.1%. Hyaluronic acid, prepared from type 28 streptococci, was furnished by Dr. Y. Ginzburg of this laboratory, glutamyl polypeptide of the anthrax bacillus was supplied by Dr. R. D. Housewright, and desoxyribonucleic acid was purchased from Nutritional Biochemicals Co. Stock solutions of the various acidic polymers were prepared in distilled water to contain 5 mg/ml. Blockage of antibacterial activity was examined when the acidic macromolecules were added to the test systems just prior to inoculation with the microorganisms. Turbidity readings were made every 2 hours for a 10 hour period, during which time the ability of any particular acidic polymer to reverse growth inhibition could be observed. Normal growth and growth inhibition controls were included with each experiment.

Preliminary experiments were carried out to examine differences in the antibacterial capacity of the thymus peptide against 2 encapsulated and non-encapsulated species. A Group A, type 28 streptococcus and a strain of *Bacillus anthracis* were tested. Thermostability of the peptide was tested in distilled water at acid, neutral and alkaline pH. The pH for greatest activity was determined using phosphate buffered broth at the pH values of 6.7 and 8.0. Electrophoretic mobility of the product was measured using cacodylate buffer (pH 7.0) at an ionic strength of 0.08. An ultraviolet absorption pattern was run using a 0.05% solution of the thymus peptide. An amino acid analysis was performed following the method of Stein and Moore(11,12). The Dowex 50-X4 resin (-400) was used in the column. The thymus peptide sample was weighed from the lyophilized state in the amount of 63.5 mg. The product was hydrolyzed in a vacuum-sealed glass tube with 3.5 ml of constant boiling hydrochloric acid for 48 hours at 110°C. The hydrolysate was carefully washed from the tube into a round bottom flask and vacuum distilled with 3 additions of distilled water. After the third distillation the residue was brought down to approximately a 1 ml volume and carefully washed into a 25 ml volumetric flask with citrate buffer, pH 3.1, and brought to volume with buffer and distilled water. The final pH of the diluted sample was 2.2. The sample was then filtered through a fiber glass pad and stored at 4°C until used. The total nucleic acid content of the thymus peptide was determined from the phosphorus value(6).

*This investigation was supported by Office of Surgeon General, Department of the Army, and National Heart Institute, N.I.H., Public Health Service.

Desoxyribonucleic acid was measured by a modification of the Dische test(14). The ribonucleic acid value was computed by subtraction of the Dische test result from the total nucleic acids. Total carbohydrate was measured by the anthrone test(13) after deproteinization with trichloroacetic acid, and was based on glucose equivalents. The total protein content of the thymus peptide was obtained from the amino acid assay and by the microKjeldahl method(9).

Results. The *in vitro* antibacterial potency of the thymus peptide was quite high. The final step in the preparation of the active fraction, which involved removal of precipitable materials at half saturation with ammonium sulfate, concentrated activity 2 to 3 times. The outgrowth of a staphylococcus was inhibited for 8 hours by 5 μg thymus peptide per ml of broth.

The acidic macromolecules blocked the antibacterial action of the tissue peptide when the ratio of acid to peptide was equal or greater. The addition of 0.5 to 1.0% NaCl to any of the test systems interfered with the capacity of the acidic polymers to block antibacterial activity of the tissue peptide. In the correlative study with encapsulated and non-encapsulated streptococci and anthrax bacilli it was found that the encapsulated forms were less strongly inhibited by the thymus peptide than the rough forms, particularly in the case of the anthrax organisms. However, the differences in degree of inhibition between smooth and rough streptococci were not always significant.

The peptide was found to be very active at pH 8.0 but its concentration had to be increased 10-fold to approach the same degree of antibacterial effectiveness at pH 6.7. From the electrophoretic determinations the material was found to migrate toward the negative pole at pH 7.0. The mobility was calculated to be 6.8×10^{-5} cm^2 volt^{-1} sec.$^{-1}$.

The peptide was active against staphylococci, Group A streptococci and anthrax bacilli but not type II pneumococci. While it inhibited *Escherichia coli, Salmonella typhosa* and *Vibrio comma* were not affected markedly by its antibacterial action. The ultraviolet absorption pattern showed one peak at 264 mμ indicating a protein-nucleic acid complex. The material could be dialyzed in cellophane bags for prolonged periods without a detectable loss in antibacterial potency. It was very heat stable, withstanding boiling temperature for periods up to one hour at neutral or acid pH. However, most of its activity was lost in 15 minutes when boiled at pH 9.6. It was quite potent against a strain of *Micrococcus pyogenes*, var. *aureus* (5-10 μg per ml) in nutrient broth but in more complex media its activity was decreased somewhat. The factor was quite soluble in water at acid or neutral pH, forming tan colored solutions at concentrations of 1-2%.

TABLE I. Amino Acid Analysis of Thymus Peptide.

Amino acid	% amino acid of total wt	g amino acid/100 g protein
Arginine	1.64	2.1
Lysine	27.10	34.7
Phenylalanine	.63	.8
Tyrosine	.34	.4
Leucine	3.20	4.1
Isoleucine	.75	.9
Valine	3.50	4.5
Alanine	13.85	17.8
Glycine	3.48	4.5
Glutamic acid	3.98	5.1
Proline	8.22	10.5
Aspartic acid	2.27	2.9
Threonine	4.10	5.4
Serine	4.24	5.4
Ammonia	.84	1.1
Total	77.25%	99.2

Table I contains the result of the amino acid analysis of the thymus peptide. The basic amino acid, lysine, represented nearly 35% of the total weight of the protein portion. The concentration of arginine was small and histidine was absent. The total protein content of the tissue peptide was found to be approximately 78% by the amino acid analysis. An average value of 80% protein was obtained by microKjeldahl determinations. Hydrolysis of nucleic acids by this latter method may have accounted for the higher value.

From the phosphorus value of 0.69%, total nucleic acids were calculated to be 6.37%. The Dische test result for desoxyribonucleic acid was 4.0% and the ribonucleic acid con-

tent, determined by subtraction, was 2.37% of the total peptide. Total carbohydrate, as glucose equivalents, was shown to be 0.83% by the anthrone method.

Discussion. Certain of the characteristics of the thymus peptide which have been studied reaffirmed and enlarged upon the original work which showed that this tissue factor was a basic polypeptide(1). The acid and heat stability of the thymus material has been confirmed. The electrophoretic mobility at pH 7.0 was found to be almost the same as that reported in the original work. The lysine content of the peptide as first reported was 29.3% by microbiological assay and in this case it was 34.7% by column chromatography. Furthermore, thymus peptide antibacterial activity was blocked by the acidic polymers, hyaluronic acid, glutamyl polypeptide and nucleic acid. Such blockage by acidic macromolecules has been reported for the previously cited thymus peptide(2,10, 15). Furthermore, the thymus peptide reported here was found to be most active at an alkaline reaction and apparently more active against gram positive bacteria.

Crampton, Stein and Moore have recently performed amino acid analyses on three calf thymus histone fractions(4). One of the 3, histone Fraction A, was shown to contain a large amount of lysine as well as alanine and proline and to be lacking four amino acids, cystine, methionine, histidine and tryptophane. The thymus peptide studied here was also rich in lysine, alanine and proline and lacking in cystine, methionine, histidine and tryptophane. It seems very likely that this peptide is the same as the histone Fraction A reported by Crampton and co-workers.

The antibacterial thymus peptide investigated by Hirsch and Dubos(7) was shown to contain large amounts of lysine and arginine. From its amino acid analysis it appears that this thymus factor is similar to Fractions B and C of Crampton, *et al.* Since the peptide was dialyzable it is possible that it represented a lower molecular weight histone fraction. It is not difficult to envisage the formation of split histone products differing in molecular weight and amino acid composition when extracting these basic proteins by related chemical procedures. The work of Weissman and Graf(16) indicates that these histone derivatives occur as a result of the splitting of the tissue nucleoproteins.

Summary. The antibacterial thymus peptide has been characterized and strong confirmatory evidence has been presented to support the view that it has been derived from a histone present in thymus tissue.

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Time and Dose Relationships in Estriol-Estradiol Interaction.* (22729)

Alan E. Wicks and Sheldon J. Segal (Introduced by R. H. Flocks)
Department of Urology, State University of Iowa, Iowa City

Biologic effects of estradiol can be modified by concurrent administration of a second estrogenic substance. Uterotrophic effect of estradiol in castrate female rats can be diminished by injecting estriol(1). Similarly, estradiol-induced pituitary enlargement can be prevented by simultaneous treatment with weaker synthetic estrogens(2). The former effect is reflected not only by organ weights but also by diminished activity of particular enzyme systems in uterine tissue(3). For interpretation of these surprising effects it should be remembered that the dose-response curve of estradiol, after having reached a maximum, declines to a lower plateau. Thus it remains to decide whether combination of two estrogens might only produce the plateau effect of more than optimal estradiol dosages, before other mechanisms are drawn into consideration. Preceding an increase in tissue dry weight, uterine tissue accumulation of fluid results from estrogenic stimulation. The onset of this fluid uptake phase is more rapid with estriol administration than with estradiol(4). In concurrent injections of both hormones, rapidly absorbed and faster acting estriol may diminish the observed response to estradiol by causing a precocious development of maximal uterine response which would pass unnoticed in a standard 3 day assay.

It is the purpose of this study to investigate the possibility of masked-summation effects of estradiol and estriol. Evidently temporal and qualitative differences in uterine response produced by various hormones may also affect the outcome of combination treatments. An analysis has been made of weight changes of uteri in immature female mice following administration of estradiol, estriol and combinations of both.

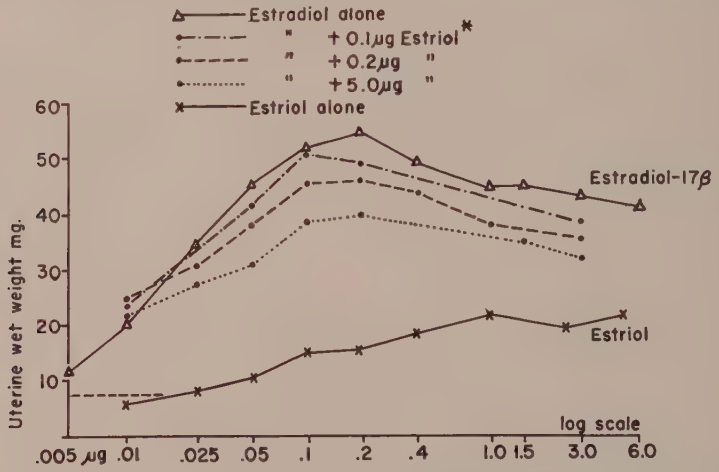


FIG. 1. Dose-response curves of estradiol-17β estriol and combinations of both. The dose of estriol is constant in each of the combination curves, as indicated in the key. Statistical computations appear in Table I.

Methods. Estrogen assays were performed according to the method of Evans, Varney and Koch(5). Dose groups of 5 to 20 immature female mice (Hamilton Laboratory strain) were injected with estradiol-17β, estriol or both, b.i.d. for 3 days. Each total

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TABLE I. Effects of Estradiol-Estriol Combinations on Uterine Wet Weight. 3-day assay.

Estradiol (μg)	Estriol (μg)	No. of animals	Mean	ε*	t†	P value
.005	—	14	11.5	.45	—	—
.01	—	24	20.6	.77	—	—
	.01-.1	10	23.7	1.10	2.31	>.02
	.2	5	23.9	1.70	1.77	>.05
	5.0	10	21.3	.74	.66	"
.025	—	10	34.6	1.24	—	—
	.2	10	30.3	1.29	2.40	>.02
	5.0	15	27.7	1.54	3.50	<.001
.05	—	61	45.3	1.14	—	—
	.01-.1	30	41.1	1.23	2.50	>.01
	.2	28	37.4	1.40	4.39	<.001
	5.0	53	30.3	.78	10.87	"
.1	—	42	51.4	1.76	—	—
	.01-.1	30	50.7	1.80	.28	>.1
	.2	20	45.5	2.46	1.95	.05
	5.0	29	38.7	1.90	4.90	<.001
.2	—	22	54.7	2.74	—	—
	.01-.1	30	48.4	1.32	2.07	>.02
	.2	20	45.7	2.14	2.59	>.01
	5.0	39	39.6	1.37	4.92	<.001
.4	—	29	48.5	2.10	—	—
	.2	10	43.4	2.94	1.41	>.05
1.0	—	39	44.5	2.23	—	—
	.2	9	37.1	3.05	1.96	>.05
1.5	—	40	44.9	1.79	—	—
	5.0	5	34.3	3.42	2.75	<.01
3.0	—	40	42.9	1.23	—	—
	.01-.1	10	38.0	2.60	1.70	>.05
	5.0	5	31.4	3.09	3.46	<.01
6.0	—	20	40.7	2.02		

$$^{*}\epsilon = \frac{\sqrt{\Sigma d^2}}{n(n-1)}. \qquad ^{\dagger}t = \frac{M_1 - M_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}.$$

dose was contained in 0.3 cc corn oil solution so that a single injection was a volume of 0.05 cc delivered from a 0.25 cc syringe. When 2 estrogens were administered each was given at a separate injection site. A control group injected with corn oil was included with each experiment. Small volumes used eliminated leakage from the needle puncture. On the fourth day, 18 hours after last injection, animals were sacrificed by cervical dislocation and uteri were freed at vagina and caudal ends of the oviducts. Uteri were blotted dry and weighed on a 50 mg Roller-Smith torsion balance. For each group receiving estradiol or mixed estrogens the mean and its standard error were calculated. Student's t test was applied using the formulae indicated in Table I, and employing the 1% confidence limit for determining significance. In some cases means represent the combined data of several experiments. Number of animals per group and pertinent statistical computations appear in Table I. In a separate set of experiments injection schedules were set up as above with 30 animals per group. At the end of 1 day, 2 days, and 3 days, 10 animals of each dosage group were sacrificed and uterine weights recorded (Table III). Dry weights of uteri were obtained by placing organs in preweighed aluminum foil pans and drying in an oven at 100°C. Usually constant final dry weight was achieved after 18 hours.

Results. Dose-response curves of estradiol and estriol (Fig. 1). A uterine weight increase of 100% above control value is produced with about .01 μg of estradiol (total 3 day dose). Maximal response occurs only with a total dose of .2 μg. At higher doses uterine weight increase remains below this maximal value. Estriol stimulates 100% increase in uterine weight at a dose of about .1 μg and an even greater increase with a total dose of 1.0 μg. Higher doses of estriol, up to 5.0 μg, do not enhance uterine stimulation. Both dose-response curves, therefore, ascend to maxima and then plateau. Slope of the ascending portion is steeper on the estradiol than on the estriol curve.

Dose-response curves of estradiol and estriol combinations (Fig. 1). Estriol inhibits the uterine response to estradiol at all dosage levels tested except .01 μg. At this point there is no significant difference between estradiol alone and any of the combination doses (Table I). Extent of inhibition increases in proportion to the estriol fraction. As a result there is a gradual decline in slopes of mixed dose-response curves; the greater amount of added estriol, the greater decline. In no case does the addition of estriol enhance the estradiol effect.

Effects of estrogen combination on uterine dry weights (Table II). The inhibiting effect described above, measured by weights of freshly dissected uteri, is not merely a matter

TABLE II. Effect of Estriol on Uterine Response to Estradiol Dry Tissue Weights—3 Day Assay.

Group*	Dose, μg	Dry wt uterus, mg
Control		1.64 ± .23†
Estriol	5.0	4.23 ± .31
Estradiol	.05	7.33 ± .34
Both	as above	5.46 ± .41‡

* 20 animals/group.
† ± stand. error.
‡ P value, vs. estradiol alone, .001.

of changed water balance but is also reflected by changes in dry weights. This is illustrated by combination of 5.0 μg estriol with .05 μg estradiol, which produces a significantly smaller gain than occurs following administration of .05 μg estradiol alone.

Daily increments in uterine weights with estrogen treatments (Table III). Daily uterine wet weight gain during administration of a total dose of .05 μg estradiol over a 3 day period is shown in Table III. Largest percentage gain occurs during the second day of treatment and a further uterine weight increase takes place during the third day. Uterine weight changes induced by a total dose of 5.0 μg estriol in 3 days follow a different course. After a slight initial gain during the first day, major portion of weight increase occurs during the second day and the third day's treatment fails to cause a further increase.

Uterine weight gain of each 24 hour period during the 3 day estradiol treatment is inhibited when estriol is given concurrently (doses of each as above). Uteri of doubly injected animals sacrificed after the first day show only 73% of weight gain resulting from estradiol alone (an inhibition of 27%). For a 2 day period expected estradiol stimulation is diminished by 42%. After completion of the 3 day injection schedule inhibition of weight gain is also 42% (Table III).

TABLE III. Daily Effects of Estrogens on Uterine Weight.

Group*	Dose, μg	Hr	Wet wt uterus, mg	Inhibition of daily wt gain, %†
Estriol	1.66	24	10.1	
Estradiol	.16	"	15.4	
Both	as above	"	13.2	27
Estriol	3.33	48	17.6	
Estradiol	.033	"	34.1	
Both	as above	"	21.9	42
Estriol	5.0	72	18.9	
Estradiol	.05	"	49.2	
Both	as above	"	30.0	42
Control		"	7.3	

* 10 animals/group.
† Estradiol-induced wt gains above control, considered 100%.

Discussion. Impeding effect of estriol occurs along the ascending segment of the estradiol dose-response curve as well as with higher doses. Extent of inhibition, using a conventional 3 day assay, is proportional to amount of estriol administered. There is no instance of an additive effect. From these observations it may be concluded that diminution of uterine weight increase is not a masked-summation effect in which two estrogens exceed the maximum estrogenic response dose. Among the dosages of estradiol employed, only .01 μg gives a response comparable to that obtained with estriol. It is of particular interest, therefore, to compare this dose of estradiol alone with a combination using an amount of estriol which causes the same uterine response (5.0 μg). In this case responses to single estrogens or to the combination are the same. These facts concerning dose relationships of estriol-estradiol interaction suggest that a competitive inhibition results in the attenuated response. Day by day analysis of uterine changes in doubly injected animals supports this view. There is no indication that combination of estriol and estradiol shifts time of maximal effect since inhibition occurs as early as the first day of treatment. Hence doubly injected animals never equal or exceed uterine response resulting from only estradiol.

In addition to estriol, several other steroids have been found to exert a limiting effect on estradiol-induced uterine stimulation in rats (6,7). Particular emphasis has been placed on the ability of adrenal cortical steroids to impede uterine response to estradiol(8). The possibility remains to be tested that estriol and other non-adrenal steroids known to inhibit estradiol stimulation of uterine growth and enzyme systems(3) act by causing release

of ACTH and secondarily adrenocorticosteroids with their modulating effect on uterine metabolism.

Summary. Uterine response to estradiol in immature female mice is inhibited significantly by concurrent injection of estriol. Extent of inhibition is proportional to amount of estriol added. Impeding interaction occurs along the ascending segment of the estradiol dose-response curve and with higher doses as well. In no case does a combination of estrogens have a positive additive action. These conclusions are based on both wet and dry uterine weight observations. Day by day analysis shows that uterine weight gain of each 24 hour period during 3 day estradiol treatment is inhibited when estriol is given concurrently. These studies negate the possibilities that estriol-estradiol interaction is a masked-summation effect in which time of maximum effect has shifted, or that an additive effect occurs in which two estrogens combined exceed maximum response dose. Rather, it appears that estriol competes actively at a physiologic site essential in development of usual estradiol stimulation of the uterus.

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Factors Influencing Host-Virus Interactions. II. Alteration of Coxsackie Virus Infection in Adult Mice by Cold.* (22730)

WILLIAM D. BORING,† GABRIELE M. ZU RHEIN, AND DUARD L. WALKER
(Introduced by C. V. Seastone)

Departments of Medical Microbiology and Pathology, University of Wisconsin Medical School, Madison, and Naval Medical Research Institute, Bethesda, Md.

Coxsackie virus infections in infant and adult mice are remarkably dissimilar(1,2,3). Many tissues, such as brain, muscle, liver, and heart, develop extensive lesions in very young animals but become refractory to the virus as mice reach 1 to 3 weeks in age. In infections with Conn. 5 strain of Coxsackie virus in adult mice the pancreas appears to be the only regular site of virus multiplication and histopathology. Even though, as a result of initial viremia, virus is available to other tissues, viral multiplication and tissue damage remain localized and non-fatal. A single injection of cortisone alters the infection with Conn. 5 virus in adult mice in such a manner that the infection becomes lethal (4) with multiplication of the virus and histopathology occurring in tissues that are unaffected in untreated animals of the same age (3). This report presents data indicating that the natural resistance of adult mice to disseminated infection with this virus can be altered by another factor, namely a cold environment, to result in rapidly and uniformly fatal disease.

Materials. Virus. The Conn. 5 strain of Coxsackie virus was used and methods for

* Opinions expressed in this article are solely those of the authors and do not necessarily reflect viewpoint of Navy Department.

† Data in this report were submitted to Graduate School, University of Wisconsin, in partial fulfillment of requirements for degree of Doctor of Philosophy. Present address: Department of Medical Microbiology and Public Health, Medical College of Georgia, Augusta.

preparation of virus suspensions, normal mouse tissue suspensions, and general methods of virus titration and neutralization tests have been described(3). In this study all infant mice 24-48 hours of age employed in a set of virus titrations were mixed and redistributed to mothers at random, and equal numbers, usually eight, were inoculated with each dilution. Unless otherwise stated, mice referred to as adult mice were 4-6 weeks of age and averaged 14-18 g in weight. Sexes were mixed at random. Immune serum was prepared by injecting mice once weekly for 3 weeks with 0.2 ml of a 1% suspension of infected infant mouse tissue. The mice were bled one week after the last injection. Normal serum was obtained from similar, but uninoculated, mice. A room varying in temperature between 23° and 27°C was used to house mice designated as held at 25°C. There was no special control over humidity which varied with atmospheric conditions. Mice referred to as held at 4°C were placed in a refrigerated room which was maintained at 3-5°C and approximately 60% relative humidity while those designated as held at 36°C were kept in room maintained at 35-37°C and approximately 12% relative humidity. Mice were housed in wire cages open to the atmosphere except for the top, bottom and the lower two inches of the sides, and were caged in groups of 8 to 12 without restrictions on huddling or activity. Food and water were available *ad libitum*.

Results. Although adult mice of strain used in this study seldom suffer apparent ill effects from even very large doses of the Conn. 5 strain of Coxsackie virus, experiments indicated that if infected mice were kept at 4°C following inoculation, the infection became uniformly lethal. A representative experiment is presented in Table I. Adult mice previously housed at 25°C were inoculated i.p. with 4000 infant LD_{50} of virus and then maintained at test temperatures along with control mice injected with normal mouse tissue suspension. By the end of fourth day all infected mice at 4°C were dead, while infected mice at 25°C, with one exception, survived the 10 day observation period. The

TABLE I. Lethal Effect of Coxsackie Virus (Conn.-5 Strain) Infection in Adult Mice at 4°C.

Inoculum (i.p.)	Environmental temp., °C	Deaths†/No. inoculated
Virus*	25	1/12
	4	10/10
	36	0/ 8
Normal mouse tissue suspension (.05 cc)	25	0/12
	4	0/ 8
	36	3/ 8

* 4000 LD_{50} (.05 cc) for infant mice.
† No. of mice dying during 10 days of observation.

one mouse dying in the 25°C groups represents the rare death seen in adult mice infected with the Conn. 5 strain. Although the infection was lethal at 4°C, infected mice placed in another stressing environment, 36°C, did not succumb. Indeed, more animals died in the control than in the test groups at this temperature. This is in keeping with numerous experiments which showed that mice of the strain employed thrived at 4°C for periods up to 3 weeks but that 36°C was a more unfavorable environment and occasional deaths occurred after one week of exposure.

To determine size of viral inoculum required to produce a lethal infection in adult mice at 4°C, comparative titrations of a virus suspension were made using adult mice held at 4°C and 24-48 hr old infant mice at 25°C. The inoculum for both groups was 0.05 ml given i.p. The LD_{50} for adult mice at 4°C was $10^{-4.5}$ and was 10 times the LD_{50}($10^{-5.5}$) for 24-48 hr old infant mice.

Additional experiments showed that lethal infection followed subcutaneous as well as intraperitoneal inoculation of virus in mice held at 4°C and that alteration of Coxsackie virus infection by a cold environment was not limited to mice 4-6 weeks of age since mice 11 weeks of age were also susceptible. The relative susceptibility of adult mice of various ages was not examined in detail.

Specifically to relate this phenomenon to an activity of the virus in infected mice held at 4°C it was important to determine whether the lethal effect could be neutralized *in vitro* with specific antiviral serum and whether pas-

TABLE II. Specific Neutralization of Coxsackie Virus and Passive Immunization of Adult Mice against Lethal Infection at 4°C.

Preliminary* inj.	Inoculum (i.p.)	Environmental temp., °C	Deaths/ No. inoc.
None	Virus† + antiserum	4	1/10
"	" + normal serum	"	9/10
Antiserum	Virus, 140 LD_{50}‡	"	0/15
"	" 280 "	"	0/15
Normal serum	" 140 "	"	13/13
" "	" 280 "	25	0/15

* 0.5 cc mouse serum given i.p. 3 hr before inoculum.
† 280 LD_{50} of virus in 0.1 cc of mouse serum.
‡ Determined in infant mice.

sive immunization of mice with specific antiserum would confer immunity to the lethal effect. Data are presented in Table II which show that death at 4°C was prevented by mixing the viral inoculum with specific immune serum prior to inoculation, and by passive immunization of mice with specific antiserum previous to viral infection.

Since it appeared possible that the lethal effect on infected mice at 4°C might have been due only to inability of mice with pancreatitis to survive in the cold, measurements of levels of virus reached in these animals were made, and the pathological lesions produced were compared with those seen in adult mice at 25°C. In Table III are presented the virus levels found in the blood and liver on the 2nd and 4th days after adult mice were inoculated with Conn. 5 virus and then maintained at either 4°C or 25°C. It is evident that in mice at 4°C a viremia persisted through the 4th day although the blood was cleared of virus by that time in mice at 25°C.

TABLE III. Levels of Virus in Tissues of Adult Mice Infected with Coxsackie Virus (Conn. 5)* and Held at 4°C or at 25°C.

Tissue	Day of infection	Virus titers† in mice at: 4°C	25°C
Blood	2	$10^{-4.5}$	$10^{-4.3}$
	4	$10^{-4.0}$	$<10^{-1.0}$
Liver	2	$10^{-4.0}$	$10^{-3.8}$
	4	$10^{-5.1}$	$<10^{-1.0}$

* Inoculum = 200 infant LD_{50} of Conn. 5 virus.
† Titers in pooled tissues of 5 adult mice as measured in suckling mice.

In the livers of mice in the cold the virus titer increased from the 2nd to the 4th day and was found at a high level on the 4th day of the infection while in mice at 25°C the virus had been reduced to a low level in the liver by the 4th day. Virus recovered from the livers of mice at 4°C was identified as the Conn. 5 strain of Coxsackie virus by neutralization tests in infant mice.

On gross examination of mice dying of Coxsackie infection at 4°C marked inflammation of perinephric, mesenteric, and pelvic fat was noted in many. In approximately 25% of the animals there appeared to be gross bleeding into the small intestine. Similar abnormalities were not found in infected mice held at 25°C nor in uninfected control mice maintained at 4°C. Microscopic examination of tissues revealed the following: *Lungs.* A slight to moderate interstitial pneumonitis was found in infected animals at both 4° and 25°C. *Heart.* In 7 of 9 infected mice held at 4° lesions were found in the myocardium. These consisted of focal areas of fiber degeneration and some of these foci contained basophilic granules. Occasionally small inflammatory granulomas consisting of fibroblasts, macrophages, and lymphocytes were seen. In contrast to these lesions observed in mice at 4°C, no myocardial lesions were found in infected mice held at 25°C. *Pancreas.* A severe pancreatitis was found in infected mice in both 4°C and 25°C groups. *Fat.* Marked enzymatic necrosis was present in the perinephric and peripancreatic fat in both the 4°C and 25°C groups of animals. Inflammatory lesions in the interscapular fat pad were similar in extent and kind in infected mice at both temperatures. *Liver.* In animals at 25° there were occasional small intralobular and portal inflammatory foci. In mice at 4° similar but more extensive lesions were found and with greater frequency. Additional features in the animals at 4° were considerably cloudy swelling, single or small groups of balloon cells, small areas of parenchymal cell necrosis and, in one animal, marked nuclear pleomorphism associated with slight to moderate numbers of mitoses. *Skeletal Muscle.* No specific abnormalities were found in skeletal mus-

TABLE IV. Relationship of Time of Exposure to 4°C and Lethal Effect of Coxsackie Virus (Conn.-5) in Adult Mice.

Environmental temperature		Deaths/No. inoculated
Before inoculation*	After inoculation†	
4°C for 2 days	25°C	0/12
" " 1 day	"	0/12
25°C	4°C, 1 day, then 25°C	0/12
"	" 2 days, "	0/12
"	4°C	12/12
"	25°C, 1 day, then 4°C	12/12
"	" 2 days, "	9/12
"	" 4 days, "	9/12
"	" 6 days, "	0/12
"	" 8 days, "	0/12

* Inoculum = 1500 infant LD_{50} given i.p. in 0.05 cc.

† Observation period = 10 days after mice were placed at 4°C.

cle at either 25°C or 4°C. *Intestine.* In all mice at 25° and most animals at 4°C no abnormalities were found in the piece of small intestine obtained for histological examination. In 2 of 9 mice at 4°C large quantities of blood were visible in the intestinal contents, confirming the gross examination finding of intestinal hemorrhage. The intestinal mucosa in one of these mice was markedly disrupted suggesting that the site of bleeding was in the small intestine. *Other tissues.* No lesions were found in the brain, kidneys, adrenal, or spleen at either temperature. No abnormalities were found in the tissues of control groups of mice injected with normal mouse tissue extracts and held at 4°C or at 25°C.

Relationship of time of exposure of mice to 4°C and lethal effect obtained with the Conn. 5 Virus. Since an environmental temperature of 4°C was effective in influencing the infection when continued exposure was begun immediately after inoculation, it was desirable to determine the time, relative to inoculation, during which cold would alter the infection and the duration of exposure necessary to result in lethal infection. In Table IV data from one of several experiments designed to explore these points are presented. Exposure of mice to 4°C for periods up to 48 hours either before or after virus inoculation was insufficient to result in fatalities. However, continued exposure of the animals to cold begun as late as 4 days after virus inoculation resulted in the death of the majority of the mice while mice survived if cold exposure was delayed until 6 or more days after infection.

Discussion. The experiments described indicate that although adult mice possess a natural resistance to the Conn. 5 strain of Coxsackie virus such that the disease is limited to a non-fatal infection of the pancreas, this refractoriness is lost when the animals are maintained in a cold environment and a lethal infection results which is characterized by a persisting viremia, high levels of virus in the liver, and lesions demonstrable in other organs as well as in the pancreas. The mechanisms by which cold reduces resistance to this virus are not yet clear although the fact that cortisone causes a similar loss of resistance suggests that cold may act through its capacity as a stress agent to cause excessive secretion of adreno-cortical steroids. That the change in resistance is due only to the non-specific effects of stress might be questioned, however, since heat, also a severe stress, does not reduce resistance. Another possibility presents itself in the well known stimulation of thyroid activity by a cold environment and depression of that activity by elevated temperatures. Evidence that changes in thyroid activity can influence resistance to bacterial and viral infection has been obtained by several investigators(5,6,7) although the direction of the change has not been constant with the several agents studied.

Reports in the literature(8,9,10) indicate that a cold environment may affect other host-virus systems although none so sharply as that studied in the experiments reported here. Cold has usually intensified the results of the infection but in some instances has afforded protection to the host. Because the effects of cold on the Conn. 5 strain Coxsackie virus infection in mice are so sharp and unequivocal we are continuing the study of this infection in an effort to further clarify the mechanisms involved.

Summary. 1. Exposure of adult mice infected with the Conn. 5 strain of Coxsackie virus to a cold environment resulted in an infection with a persisting viremia, high levels

of virus in the liver, pathologic lesions in other organs as well as in the pancreas, and a uniformly fatal outcome. 2. A lethal infection was prevented by neutralization of the virus or passive immunization of adult mice with specific antiserum. 3. The LD_{50} for adult mice at 4°C was approximately ten times that for suckling mice at 25°C. 4. It was demonstrated that to initiate a fatal infection exposure to cold had to be continuous and begun within a few days after virus inoculation.

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Effects of Short Chain Fatty Acids on Hepatic Acetate Metabolism in Cold Exposure and Fasting.* (22731)

E. J. MASORO AND SYLVIA S. PANAGOS†

Department of Physiology, Tufts University School of Medicine, Boston, Mass.

We reported that liver slices prepared from rats fasted for a period of 24 hours at an environmental temperature of 0-2°C ("cold-fasted" rats) showed severe depression in oxidation of acetate-1-C^{14} to $C^{14}O_2$(1). This defect in acetate oxidation could be restored to normal by addition of glucose(1) or pyruvate(2) to the incubation medium. The evidence suggested that this effect of glucose and pyruvate must be related to a metabolic event prior to entry into the Krebs cycle because succinate was much less effective than pyruvate and glucose in reversing this metabolic defect while citrate and α-ketoglutarate were without effect at all.

Since phases of carbohydrate metabolism other than the Krebs cycle are involved, it is important to know whether acetate oxidation is specifically linked to carbohydrate metabolism or whether other substances can serve as energy sources capable of reversing the block in acetate oxidation imposed by cold exposure and fasting. The latter possibility was tested by adding short chain fatty acids to the incubation medium. The results of these experiments are reported below.

Methods. Adult male rats of Wistar strain were allowed to eat, *ad libitum* for 7 to 10 days, a diet composed of 25 g casein, 10 g fat, 51 g dextrose, 3 g salt mix(3), 5 g cellulose, 6 g Brewer's yeast, 0.04 g cod liver oil concentrate and 0.01 g α-tocopherol. Following dietary stabilization the rats were either given the same diet, *ad libitum*, at 25°C or were fasted for 24 hours at 0-2°C. The animals were killed by blow on head. The liver was rapidly excised and placed in a beaker containing ice-cold Krebs-Henseleit bicarbonate buffer solution(4). Tissue slices were prepared free-hand with razor blade and these slices were collected in petri dish containing ice-cold buffer. The liver slices were blotted free of excess solution on filter paper. Approximately 250 mg of tissue were weighed out and then transferred to specially designed

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† Predoctoral Fellow of National Science Foundation.

incubation flasks(5). All incubations were carried on in duplicate flasks. The main compartment of the incubation flasks contained 250 mg of liver slices, 4.5 ml of Krebs-Henseleit bicarbonate buffer and 0.5 ml of the solution containing the acetate-1-C^{14} as substrate, at concentration in all cases of 1 x 10^{-3} M. When unlabeled substrates were also present, they were neutralized to pH 7.4 with sodium hydroxide and dissolved in the buffer. The system was incubated for 3 hours at 37.5°C with continuous shaking. Total lipids (fatty acids and unsaponifiable materials) were extracted from liver slices by the method of Baruch and Chaikoff(5). An aliquot of the chloroform extract (containing the total lipids) was used for determination of cholesterol digitonide using the method of Sperry and Webb(6). The total C^{14} content of fatty acids was calculated by subtracting the C^{14} content of the cholesterol digitonide from the C^{14} content of the total lipid fraction. The C^{14} content of the lipids and the cholesterol digitonide was determined by methods already described(7). The $C^{14}O_2$ formed during the incubation period was collected by the method of Chernick *et al.*(8) and counted in the form of $BaC^{14}O_3$ by the method of Entenman *et al.*(9). The acetate-C^{14} content was determined by methods described in an earlier paper(7). The unlabeled short chain fatty acids were purchased from commercial sources and were of the highest purity obtainable. Each was further purified by fractional distillation; a Widmer fractionating column was used in these distillations.

Results. The term "control" rat designates rats allowed to eat *ad libitum* at 25°C while "cold-fasted" rat describes rats fasted at 0-2°C for 24 hours.

Liver slices from control rats oxidized 34 ± 0.7% of the acetate-1-C^{14} to CO_2, converted 6.7 ± 0.84% to fatty acids and 0.5 ± 0.07% to cholesterol (Table I). In the case of experiments with control rats, the liver slices were prepared from the same group of rats that were used for the "cold-fasted" experiments and the slices were incubated at the same time and under identical conditions as those of the "cold-fasted" rats to be described below.

TABLE I. Acetate Metabolism by Liver Slices from Control Rats.

No. of rats	% conversion* of acetate-1-C^{14} to		
	CO_2	Fatty acids	Cholesterol
15	34 ± .7†	6.7 ± .84	.5 ± .07

* % of acetate-1-C^{14} converted to CO_2, fatty acids and cholesterol is calculated for a system consisting of 500 mg of liver slices in 10 ml of buffer solution containing 1 × 10^{-3}M sodium acetate-1-C^{14}; the incubation flasks were shaken in water bath at 37.5°C for 3 hr.

† Stand. dev. of mean.

The effects that certain short chain fatty acids have on hepatic acetate metabolism of the "cold-fasted" rats are recorded in Table II. Liver slices from the same rat were incubated with and without the further addition of unlabeled substrate in concentrations noted in this table.

Propionate was added to the incubation medium at 4 x 10^{-3} or 8 x 10^{-3} M concentrations; at neither concentration did it restore the capacity of these slices to oxidize acetate-1-C^{14} to $C^{14}O_2$ but rather further reduced the extent of this conversion. Propionate had little or no stimulating effect on the lipogenic and cholesterogenic activity of these tissues.

Addition of unlabeled butyrate also further depressed acetate oxidation of the "cold-fasted" livers rather than stimulating it, and as with propionate there was no effect on the ability to convert acetate to lipids.

Since caproate is an even-numbered short chain fatty acid, it might be expected to have an action similar to butyrate. Such was found to be the case.

It was shown by Atchley(10) that isobutyrate can be converted to propionate by mammalian mitochondrial preparations; we therefore might expect that isobutyrate would behave in a manner similar to propionate. This proved to be true.

Acetoacetate at 4 x 10^{-3} M and 8 x 10^{-3} M concentrations not only did not stimulate acetate lipogenesis and cholesterogenesis, but, unlike the short-chain fatty acids, had no effect on $C^{14}O_2$ production as well.

Discussion. The fact that butyrate and caproate failed to reverse the block in acetate

TABLE II. Acetate Metabolism by Liver Slices from "Cold-Fasted" Rats.

No. of rats	Unlabeled substrate: Compound	Unlabeled substrate: Conc. × 10^{-3}M	% conversion* of acetate-1-C^{14} to: CO_2	Fatty acids	Cholesterol
4	None	0	8 ± 1.7†	.1 ± .00	.1 ± .04
	Propionate	4	2 ± .6	.1 ± .00	.0 ± .03
4	None	0	8 ± 1.7	.1 ± .00	.1 ± .04
	Propionate	8	1 ± .4	.1 ± .03	.0 ± .03
2	None	0	14 ± 1.0	.1 ± .02	.0 ± .03
	Butyrate	4	4 ± .5	.1 ± .02	.0 ± .02
9	None	0	9 ± .7	.1 ± .00	.0 ± .00
	Isobutyrate	4	3 ± .7	.1 ± .00	.0 ± .00
4	None	0	12 ± 2.9	.1 ± .00	.1 ± .00
	Caproate	4	5 ± .7	.1 ± .07	.1 ± .07
2	None	0	16 ± 4.5	.1 ± .00	.1 ± .07
	Acetoacetate	4	16 ± 4.2	.1 ± .00	.0 ± .00
2	None	0	16 ± 4.5	.1 ± .00	.1 ± .07
	Acetoacetate	8	13 ± 2.6	.1 ± .00	.0 ± .00

* Refer to footnote, Table I. † Stand. dev. of mean.

oxidation by the "cold-fasted" liver strongly suggests that fatty acid metabolism is not linked with acetate oxidation in the way that glucose and pyruvate metabolism are. Since it was found that butyrate is oxidized by the "cold-fasted" liver at high rates (unpublished), it is apparent that merely the lack of an adequate energy source is not the factor in the "cold-fasted" liver responsible for the low rate of acetate oxidation.

We found it particularly surprising that propionate failed to stimulate acetate oxidation in "cold-fasted" livers since it has long been known that propionate is a glucogenic compound capable of forming considerable amounts of glucose and pyruvate. However, it has recently been reported that propionate does not directly form a carbohydrate intermediate but rather that it enters the carbohydrate schema via the Krebs cycle (by forming succinate) (11). We have already shown that Krebs cycle intermediates (2) are not efficient in reversing the block in acetate oxidation of hepatic tissue from "cold-fasted" rats. We have also demonstrated (2) that relatively large amounts of pyruvate and glucose must be metabolized to reverse this block in acetate oxidation; it is therefore probable that neither Krebs cycle intermediates nor propionate are capable of forming sufficient quantities of carbohydrates and carbohydrate intermediates to effect acetate metabolism appreciably.

In each case there was a reduction in $C^{14}O_2$ formation in the presence of short chain fatty acids; a fact which suggested that those compounds may have an inhibitory action on acetate metabolism. This point has been the subject of an extensive investigation and will be considered elsewhere.

Summary and conclusions. Butyrate, isobutyrate, propionate, caproate, and acetoacetate failed to reverse the block in acetate-1-C^{14} oxidation found in liver slices from rats fasted at 0-2°C for 24 hours. It appears that the ability of glucose and pyruvate to increase the acetate oxidation activity of these liver slices is related to a specific step in carbohydrate metabolism rather than solely to the fact that carbohydrates are excellent sources of energy.

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Blood Levels of 17-Hydroxycorticosteroids in Hyperthermic Dogs.* (22732)

GEORGE BARLOW, HELMER P. AGERSBORG, JR., AND HARMON E. KEYS
(Introduced by Richard R. Overman)

Clinical Physiology Laboratories, University of Tennessee College of Medicine, Memphis

That stress produces an elevation in the level of 17-hydroxycorticosteroids has been indicated by a number of publications(1-5). These observations have ranged from noting the decreased quantity of cholesterol present in the adrenal glands of animals subjected to hemorrhage, to direct measurements of plasma 17-hydroxycorticosteroids in animals and man subjected to surgical, bacterial pyrogen, E.C.T., and whole body radiation procedures. Recently Eik-Nes has reported by abstract, that dogs given an intravenous pyrogenic dose of bacterial mucopolysaccharides exhibited prolonged elevations in plasma 17-hydroxycorticosteroids. The adrenal cortical hormone(s) are known to exert considerable influence not only on the bodily economy of fluids and electrolytes, but also on the partition of these substances. Overman *et al.*(6) have shown that in man and the monkey, marked alterations in Na and K distribution result from prolonged exposure to a febrile condition having its origin in the malarious state. It was suggested that this is indicative of an increased level of certain adrenal cortical hormones in the body.

Since little of a correlative nature is known concerning the stressful hyperpyrexic state, efforts have been made in this laboratory to understand the aberrant physiology of fever. The report of one facet of that investigation —concerning alterations in plasma concentrations of 17-hydroxycorticosteroids, of Na and of K—is the principal concern of this communication.

Materials and methods. Twenty-four healthy mongrel dogs of both sexes and weighing between 10 and 22 kg were subjected to exogenous hyperthermia. Before the animals were placed in the hypertherm cabinet but *after* the administration of intraperitoneal pentobarbital sodium (1 ml of 7% solution/5 lbs. body weight) and after placing of the arterial cannulae, control determinations were made of plasma and R.B.C. electrolytes, rectal temperature and plasma steroid concentrations. Na and K were determined by flame photometry. Rectal temperatures were taken 4 inches proximal to the anus using a thermistor probe. Plasma 17-hydroxycorticosteroids were determined by the method of Nelson and Samuels(7). The dogs were exposed to an ambient temperature of 50°C, generated by shielded infrared heat lamps, for periods sufficient to provoke a rise in rectal temperature to 42.5°C. This temperature was maintained for one hour. All blood samples were obtained from a cannulated carotid artery and were heparinized. Ten dogs, treated in a manner similar to that of the fever experiments with regard to anesthesia, minor surgery and elapsed experimental time, were utilized as controls. The survival of experimental animals was found to be less than 10% 24 hours after heating.

Results. The values obtained from the analysis of plasma for 17-hydroxycorticosteroids are presented in Table I. It will be noted

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TABLE I. Control and Experimental Plasma 17-Hydroxycorticosteroid Concentrations (μg %) in Dogs Subjected to Hyperpyrexia.

Control, 37.5°C	39.4°C	At 42.5°C	42.5°C + 0.5 hr	42.5°C + 1.0 hr	1.5 hr after cabinet removal
3		2		5	
2		5		5	
3		6		28	
3	11	8		18	
3	4	3	11	9	
4	9	13	9	11	
7	12	12	14	14	
5	0	7	10	4	
3*	5	8	22	21	
0*	3	5	11	8	
2*	5	5	11	11	
2*	3	3	9	10	
0*	1	3	8	18	
0*		1			
0*		6			
6*				16	
6*				17	
8*				25	
7*				28	36
6*				17	15
4*				16	28
4*				9	12
8*				27	32
2*				17	28
			Avg		
4	5	6	12	15	25
			% change		
	+25	+50	+200	+275	+525

* Blood removed from these dogs was replaced from donor dogs.

that after the rectal temperatures had been increased from the average control of 37.5°C to 42.5°C and maintained at that level for one hour, plasma cortical steroid level had risen from 4 to 15 μg/100 ml plasma. Observations were made at the following times during the experimental period: at a point half way between the control rectal temperature and 42.5°C (39.4°C in Table I), after the temperature had reached 42.5°C, after one half hour at 42.5°C, and after one hour at 42.5°C. In addition, plasma steroid concentrations were measured in 6 dogs about 1.5 hours after removal from the cabinet (average rectal temperature, 39.4°C).

Fig. 1 shows that during the first 120 minutes, both experimental and control animals presented increments in circulating plasma 17-hydroxycorticosteroids of 2 and 3 μg % respectively. During this interval the control animals' rectal temperatures decreased an average of 1.5°C from their control value (38.8°C). It is conceivable that the hypothermic tendency produced this increment in the control animals; no comparable decrease in rectal temperature occurred in the experimental dogs. After 120 minutes, however, the course of the 2 groups of dogs became quite divergent, with those exposed to elevated rectal temperatures exhibiting marked increments in circulating plasma 17-hydroxycorticosteroid levels (Fig. 1). It was feared that the bleeding necessary to obtain adequate plasma for electrolyte and 17-hydroxycorticosteroid determinations (approximately 35 ml of whole blood), in combination with marked diminution in circulating plasma volume as reported by Agersborg *et al.*† might augment the stress produced by the hyperthermic state. Consequently, the volume of blood removed for analysis in the last 16 animals presented in Table I was replaced from healthy, normal, donor dogs. It will be seen that similar increments in plasma 17-hydroxycorticosteroids occurred despite the replacement.

The average steroid level of the 6 dogs after removal from the hypertherm cabinet was 25 μg/100 ml plasma in contrast to only 4 μg % in the controls (Table I, Fig. 1). Removal of the external stimulus apparently does not reverse the tendency for increasingly augmented 17-hydroxycorticosteroid levels.

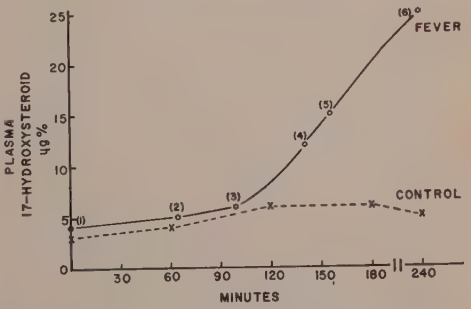


FIG. 1. Each point represents the avg of 6 or more determinations: (1) Avg control rectal temp., 37.5°C. (2) Avg rectal temp., 39.4°C; p = 0.5. (3) Rectal temp., 42.5°C; p = 0.05. (4) Rectal temp., 42.5°C; p = <0.001. (5) Rectal temp., 42.5°C; p = <0.001. (6) Avg rectal temp., 39.5° C; p = <0.001.

† Abstract

Plasma K concentrations were increased from control values of 3.9 to 5.3 mEq/l at the end of the experimental period ($p<0.001$). In general, plasma Na levels were not altered. However, the erythrocyte Na concentration was increased from a control value of 111 mEq/l to an experimental average of 115 mEq/l which is significant at the 1% level. In addition, red blood cell K concentration increased.

Discussion. The data show that severe hyperthermia produces a marked rise in the circulating level of 17-hydroxycorticosteroids in anesthetized dogs. It cannot be ascertained from these data what mechanisms are responsible for such pronounced increments; however, judging from the control determinations, anesthesia, minor surgical trauma and duration of experiment cannot be indicted. Several other possibilities might be suggested.

1) It is possible that thermal stimulation of adrenal cortical function occurs either directly or via ACTH.

2) The rate of hormone destruction in the liver might be impaired. It has been shown by Tyler *et al.*(8) and Sandberg and his colleagues(1) that during surgical stress the adrenal cortex is only submaximally stimulated, and the resulting elevation of corticosteroids results from a decreased ability of the liver to remove 17-hydroxycorticosteroids from the plasma. In this regard two modes of action are distinct possibilities in animals subjected to hyperpyrexia: a) the elevated body temperature might reduce the capacity of liver cells to conjugate 17-hydroxycorticosteroids, and b) the altered circulation of these dogs might contribute to the inability of the liver to handle adrenal steroids in a normal manner. Numerous observations of hemodynamics in hyperpyrexic dogs in this laboratory have shown marked skin dilation associated with measured rises in peripheral resistance. This suggests visceral vasoconstriction which might well decrease liver blood flow. Thus, while liver cells may still be functional or even hyperactive in the face of thermal stimulation, a serious reduction in liver blood flow could result in diminished removal of 17-hydroxycorticosteroids from plasma.

3) There is a possibility that a decreased rate of utilization or destruction could occur in the peripheral tissues of the body, but no means has yet been devised to determine either rate of utilization or destruction of adrenal cortical hormone(s) by tissue cells.

4) The increased plasma steroid levels might result from a concentration effect due to diminished plasma volume. Consideration of a typical animal in which the plasma 17-hydroxycorticosteroid level rose from 6 to 17 μg % during the experimental period reveals that such is not the case. With a control plasma volume of 1015 cc, this dog had 61 μg of circulating 17-hydroxycorticosteroids. At the end of the febrile period, while the plasma volume had been reduced to 791 cc, the total amount of circulating 17-hydroxycorticosteroids equalled 134 μg. If concentration (due to plasma volume reduction) were alone operative, one would expect very little, if any, increment in total circulating plasma 17-hydroxycorticosteroids. The observed increase of 120% in total circulating steroid level cannot be reconciled on the basis of a concentrating mechanism. As a result of these findings, it is suggested that the observed hyper-17-hydroxycorticosteroidemia is due to (1) the possibility of thermal stimulation of the adrenal cortex, (2) diminished rate of hormone destruction in the liver, (3) decreased peripheral tissue utilization, or (4) some subtle combination of these mechanisms.

The electrolyte alterations encountered were all statistically significant at the 2.5% level or lower with the exception of plasma Na concentrations. The marked increase in plasma K concentration ($p<0.001$) is deserving of commentary. Either a concentration mechanism is operative in this instance which is not readily apparent from plasma Na values, or perhaps the plasma K concentration is elevated through the release of adrenaline. Numerous investigators(9-11) have reported that the injection of adrenaline results in the elevation of plasma K concentration, and although the site of release of this K remains unknown, it has been generally attributed to the glycogenolytic influence of

TABLE II. Plasma and Erythrocyte Na and K Concentrations Obtained before and after Dogs Were Subjected to Rectal Temperature of 42.5°C for One Hour.

	Plasma electrolytes, mEq/l				Erythrocyte electrolytes, mEq/l			
	Control		Exp.		Control		Exp.	
	Na	K	Na	K	Na	K	Na	K
	154	3.9	151	6.3				
	154	3.9	151	6.7				
	146	3.9	147	4.0	99	4.2	115	5.0
	141	3.7	144	5.3	106	6.1	118	6.8
	144	4.0	145	5.4	111	6.0	113	6.4
	141	4.2	144	6.2	118	5.4	119	5.7
	146	3.9	144	7.6	115	5.3	116	5.6
	139	3.4	139	4.7	107	6.6	114	5.8
	134	4.0	138	6.0				
	140	3.5	140	4.7	113	4.0	118	4.5
	143	3.7	143	6.8	108	3.9	111	5.0
	148	3.3	145	4.6	115	5.4	113	5.1
	140	4.0	142	5.0	106	5.0	109	5.4
	149	3.8	148	4.7	110	6.1	120	6.2
	150	4.2	151	4.4	118	5.9	107	5.4
	149	4.4	150	4.8	109	6.3	119	6.9
	149	4.2	149	5.1	115	5.7	119	5.5
	150	4.0	149	5.8	112	5.8	115	6.7
	154	3.5	152	3.6				
	143	3.9	145	4.7	112	4.8	118	6.1
	145	4.1	147	4.9	112	5.3	116	5.4
Avg	146	3.9	146	5.3	111	5.4	115	5.8

adrenaline in the liver.

A consideration of 6 dogs in which plasma volume measurements were made shows that no change in total circulating plasma K occurs after the hyperthermic experience. This would appear to offer concrete evidence of a concentrating mechanism. However, in these experiments, transfer of K occurred into erythrocytes (Table II) which may have contributed to the absence of elevated total circulating K. The magnitude of the K uptake by the red blood cells is not too clear from these studies and a more complete treatment must await thorough investigations concerning alterations in the amount of cell ion. Preliminary determinations indicate that the magnitude of increase in the *amount* of erythrocyte K is quite sizeable (c. 190%).

With regard to plasma volume reductions it should be pointed out that the plasma represents the most labile fraction of the body fluid. Since the electrolyte concentrations are essentially similar on either side of the endothelial barrier, the increment in plasma K concentration would be reflected in the interstitial fluid. It seems unlikely, however, that the reduction of the plasma volume would be accompanied by an equivalent decrement in the larger and more stable interstitial fluid compartment. Therefore, the distinct possibility remains that an actual increment existed in the amount of extracellular K in these animals. The point to be emphasized here is that in many instances it is difficult to evaluate the real meaning of electrolyte alterations on the basis of altered plasma concentration alone. Such considerations apply equally to all plasma constituents. Thus, these experiments give us no real measure of the absolute amount of extracellular K, and one can only surmise that it has increased.

With these considerations in mind it would be well to regard the respiratory musculature as a possible source of some of the K. It has been repeatedly shown that muscular activity promotes loss of much cell K, and that such loss is proportional to the strength and duration of contraction(10). All animals used in these studies had very rapid breathing rates (>250/min) many of which exceeded the ability of the investigators to count by palpation, and all of which involved not only the thoracic musculature, but also the accessory respiratory muscles of the abdomen. It is therefore suggested that marked muscular effort might materially add to the extracellular K levels.

Likewise, in the face of dehydration it was noted that no change occurred in plasma Na concentration (Table II). However, the total amount of circulating Na diminished from an average of 114 meq to 91 meq in the 6 animals in which plasma volumes were measured. Clearly Na is being lost from the vascular system and the data presented in Table II would indicate that some of it is making an appearance in the erythrocytes. It is also conceivable that Na is moving into respiratory muscle cells in exchange for K, but such measurements have not been made in this experiment. The transfer of Na into muscle and red blood cells could account for the lack of rise in plasma Na concentration seen at the end of the experimental period instead of an increased concentration as might be expected

to result from the loss of water. That increments in erythrocyte Na occurred is clear, and while it is very difficult to assess the importance of alterations in canine red blood cell electrolytes, because their chemical anatomy is quite different from other body cells, such increases could be mediated by the influence of excessive amounts of 17-hydroxycorticosteroids on membrane permeability.

Summary. Twenty-four dogs were subjected to an ambient temperature of sufficient magnitude to elevate rectal temperatures to 42.5°C for one hour. These animals were studied with regard to plasma 17-hydroxycorticosteroid concentration and plasma and erythrocyte electrolyte levels. Significant changes were observed in all measured variables with the exception of plasma Na concentration, which was not altered from the control value. The physiological implications of these data are discussed.

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Increased Activity of Renal Glutaminases in Guinea Pig Following Prolonged Administration of Acid or Alkali.* (22733)

LEON GOLDSTEIN,† R. RICHTERICH-VAN BAERLE,‡ AND EARL H. DEARBORN§

Department of Pharmacology, Boston University School of Medicine, Boston, Mass.

The relationship between ammonia excretion and renal glutaminase activity has attracted much attention in recent years. Davies(1) discovered a marked increase in the ability of kidney slices from chronically acidotic rats to deaminate glutamine and other amino acids. On the other hand, Handler(2) found no increased hydrolysis of glutamine in slices from rats chronically treated with acid or alkali. White(3) observed no increase in renal glutaminase activity in homogenates from acidotic rats, while Rector(4) demonstrated a sharp day to day rise in the glutaminase activity of kidney homogenates of rats treated with ammonium chloride. Contrary to observations in man, dog and rat, we observed that, in the guinea pig, not only does ammonia excretion increase in acute acidosis but also in acute alkalosis(5). It was thought that investigation of the activity of renal glutaminases in the two conditions might aid in elucidating the biochemical mechanisms responsible for the increased urinary ammonia excretion. As a basis for work on the enzymatic mechanism of ammonia production, it was mandatory to characterize the enzymes involved. This had not been done previously and might explain the controversial observations. We have been able to distinguish three enzymes involved in glutamine metabolism in

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† Gillette Fellow in Pharmacology.

‡ Present address, Medizinische Universitätsklinik, Bürgerspital, Basel, Switzerland.

§ Present address, Research Division, American Cyanamid Co., Pearl River, N. Y.

the renal tubular cells‖: *Glutaminase I* is a hydrolyzing enzyme with an optimal pH of 7.4, is activated by phosphate and is inhibited by Sulfobromophthalein or Quinacrine (6). *Glutaminase II* is a hydrolyzing enzyme system with an optimal pH of 8.8, is dependent on the presence of α-keto acids, and is similar to the glutamine transaminase-deamidase of the liver studied extensively by Meister(7). *Glutamine synthesizing enzyme system* forms glutamine from glutamic acid and ammonia, has an optimal pH of 7.4 in homogenates, is dependent on adenosinetriphosphate and magnesium ions, and is inhibited by p-chloromercuribenzoate(7). Since it is not known which of these enzymes are involved in ammonia formation, all were investigated in this study.

Materials and methods. Adult guinea pigs, averaging 450 g (400-485 g), in groups of 3 males and 3 females each were treated for a period of 3 weeks as follows: Each group was intubated daily with either 3 ml of 0.9% sodium chloride, 3 ml 1 M ammonium chloride, or 3 ml 1 M sodium bicarbonate. The latter 2 groups received either 0.5 to 1% ammonium chloride or 1 to 2% sodium bicarbonate respectively in their drinking water. All animals were fed Purina Rabbit Chow Checkers supplemented daily with cabbage. The animals were killed by a blow on the head. The kidneys were removed, decapsulated, chilled, and made into a 5% homogenate according to the procedure of Potter(8). The enzyme assays were carried out under optimal conditions as established by previous experiments. The following incubation mixtures were used: *Glutaminase I*: .1 ml 5% tissue homogenate, .1 ml .1 M l-glutamine. .05 ml 1 M sodium phosphate (pH 7.4), .2 ml .05 M 2-amino-2 - hydroxymethyl-1,3 - propandiol (tris buffer) (pH 7.4), .9% sodium chloride to make 1 ml. *Glutaminase II*: .3 ml 5% tissue homogenate, .1 ml .1 M l-glutamine, .1 ml .5 M sodium pyruvate, .2 ml .1 M Veronal-HCl buffer (pH 8.8), .9% sodium chloride to make 1 ml. *Glutamine synthesizing enzyme*: .3 ml 5% tissue homogenate, .2 ml .5 M sodium l-glutamate, .2 ml 2.94 mM ammonium chloride, .1 ml .8 M magnesium sulfate, .2 ml .05 M adenosinetriphosphate-sodium (ATP), .2 ml .05 M tris buffer (pH 7.4), 0.9% sodium chloride to make 1.5 ml. All incubations were carried out in 25 ml serum bottles at 37.5°C for 30 minutes. At the end of the incubation period, the bottles were stoppered, injected through the stopper with 1 ml 20% sodium carbonate and analyzed for ammonia by the micro-diffusion method of Seligson(9). Glutaminase I activity was derived by taking the difference between ammonia liberated in the presence and in the absence of phosphate and corrected for the phosphate-activated, non-enzymatic decomposition of glutamine. For Glutaminase II activity, the assay results in the absence of pyruvate were subtracted from those obtained in the presence of pyruvate. Glutamine synthesis was calculated from ammonia disappearance during incubation and corrected for preformed ammonia and ammonia liberated from endogenous substrates. All results are expressed as μM ammonia liberated per 100 mg desoxyribosenucleic acid, as suggested by Knox(10) since it reflects the number of cells present. The results are similar when wet or dry weight is used as a base but show somewhat greater variability. Desoxyribosenucleic (DNA) and ribosenucleic (RNA) acids were determined on a sample of the original homogenate. The method employed for DNA was a combination of the technic developed by Schneider(11) and of Dische's color reaction for desoxyribose(12). The method of Mejbaum(13) was used to determine RNA.

Results. The percent increase in body weight of the acidotic animals was significantly less than the control or alkali treated groups. The renal DNA concentration was lower in the alkalotic animals than in the controls (Table I). Since the kidney weights were essentially equal, cytoplasmic mass must have been greater in the kidney cells of the alkalotic animals than in the controls. Renal RNA concentration was similar in all 3 groups.

To justify the statement that an increase in enzyme activity is due to induction, the following criteria must be fulfilled(14,15).

‖ Unpublished data.

TABLE I. Effect of 3 Weeks Administration of Acid or Alkali on Body Weight, Kidney Weight and Concentration of Desoxyribosenucleic and Ribosenucleic Acids in Kidney of Guinea Pig.

Group (No. of animals)	Body wt		Kidney wt		Nucleic acids	
	Increase, %	Final, g	% body wt	g	DNA mg/1000 mg d.w.‡	RNA mg/1000 mg d.w.‡
Control (6)	20 ± 3†	533 ± 17	.79 ± .03	4.1 ± .3	45.0 ± 3.2	4.8 ± .3
Acidotic (6)	*10 ± 3	*476 ± 10	.86 ± .04	4.0 ± .2	43.2 ± 3.3	4.6 ± .2
Alkalotic (5)	23 ± 2	528 ± 26	.83 ± .04	4.3 ± .3	*36.9 ± 1.6	4.2 ± .2

* Differs from controls, $P < .05$. † Mean ± S.E. ‡ Dry wt.

First, there must be an increase in enzyme concentration. To rule out the effects of altered permeability, cofactors, etc., homogenates should be employed for assays. Second, to eliminate an increase in enzyme activity due to an increased number of cells, enzyme activity should be expressed on a per cell basis. Since the DNA content of each cell of a species is relatively constant, enzyme activity is best expressed in terms of DNA. Third, since enzyme induction is a form of protein synthesis, it is dependent on amino acid and energy supplies and can be blocked by amino acid antimetabolites or agents blocking oxidative phosphorylation. In the present study the first two criteria have been fulfilled, while the third will be studied in a succeeding investigation.

Increased renal glutaminase activity occurred in the alkalotic as well as in the acidotic group (Table II). Activities of Glutaminase I, Glutaminase II, and the glutamine synthesizing enzyme were elevated in alkalosis and Glutaminase I in acidosis. The scattering of data with reference to Glutaminase I in alkalosis and Glutaminase II in acidosis was conspicuous and may, in some way, be connected with the metabolic alterations produced by the two conditions. The increase of Glutaminase I in acidosis is in agreement with the observations of Davies (1), who employed slices, and Rector (4) who used homogenates of kidneys from rats treated with acid. The contradictory results of Handler (2) and White (3) are probably due to the small number of animals used (only one or two acidotic animals were investigated) and the equivocal assay methods employed (non-optimal conditions).

TABLE II. Effect of Prolonged Administration of Acid and Alkali on Renal Glutaminases.

Group (No. of animals)	Glutaminase I*	Glutaminase II	Glutamine synthesizing enzyme
Control (6)	47 ± 10†	104 ± 9	98 ± 22
Acidotic (6)	§ 96 ± 12	140 ± 30	159 ± 24
Alkalotic (5)	‡141 ± 41	§172 ± 12	‡189 ± 29

* All enzyme activities expressed as μM NH_3 liberated or disappearing (glutamine synthesizing enzyme) from the incubation medium per 100 mg DNA per 30 min.
† Mean ± S.E.
‡ Statistically different from controls ($P < .05$).
§ *Idem* ($P < .01$).

The observation that glutaminase activity in homogenates from alkalotic animals is higher than the controls differs from the observation of Davies (1) that the activity of this enzyme in slice preparations from alkali treated animals was lower than the controls. He, however, employed slices whereas homogenates were used in the present study. Slices not only hydrolyze, but even in the absence of added ATP, synthesize glutamine. Since our studies show for the first time an increased glutamine synthesizing enzyme activity following prolonged administration of alkali, the decreased formation of ammonia from glutamine which Davies found may have been a consequence of assaying for glutaminase in the presence of increased glutamine synthesizing enzyme. Glutaminase II activity, which was observed in the present study to be increased by treatment with alkali, was not studied by Davies.

Whether glutamine from the blood is the only substrate for ammonia production or whether glutamine synthesized intracellularly is an additional source of ammonia is not known. Since both acidosis and alkalosis stimulate the ammonia production and in-

crease the glutaminase activity of the kidney, it is possible that prolonged treatment with either acid or alkali could diminish both blood and renal glutamine stores and increase the glutamate available to the synthesizing enzyme. This in turn would stimulate the synthesis of glutamine by the kidney and lead to increased activity of the glutamine synthesizing enzyme.

Summary. Increased activity of the enzymes involved in ammonia metabolism in the kidneys of guinea pigs was produced by prolonged treatment with acid or alkali. When the results were expressed on a per cell basis, Glutaminase I was increased in acidosis while in alkalosis Glutaminases I and II and the Glutamine synthesizing enzyme were increased. These increases in activity are suggestive of enzyme induction though all the criteria for this process have not yet been fulfilled.

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Lipide Analysis of Cellular Fractions in Liver Necrosis.* (22734)

W. E. CORNATZER AND DUANE G. GALLO

Department of Biochemistry, and Guy and Bertha Ireland Research Laboratory, University of North Dakota Medical School, Grand Forks

Within the past few years considerable information has been obtained concerning the pathogenesis of necrotic liver degeneration. It may be produced by dietary(1), chemical (2), or virus(3) means. The biochemical changes occurring in the liver cell during the production of necrosis are probably different for each agent. The necrotic livers of rats fed a *Torula* yeast diet are characterized by a progressive failure of oxygen consumption, ketogenesis, lipogenesis and oxidation of labeled acetate to CO_2(4). Popper *et al.*(5,6) have observed that necrosis caused by bromobenzene administration produces a decrease in the concentration of liver esterase, succinic dehydrogenase and total lipides. However, no analyses were made on the phospholipide content of the liver. Biochemical detoxification of bromobenzene causes depletion of cysteine and its precursors such as methionine from the liver(5). Koch-Weser *et al.*(7) have suggested that the condition produced in the bromobenzene treated rats is an example of a chemical conditional amino acid deficiency. In view of the above findings, experiments were undertaken to determine the lipide content of the various cellular fractions of the liver following bromobenzene administration to ascertain whether the lecithins and cephalins are involved in this process.

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Experimental. Male albino rats of the

Wistar strain weighing 100-110 g were maintained on a Purina stock diet. Acute liver necrosis was produced within 48 hours by the injection of bromobenzene, utilizing the method of Koch-Weser *et al.*(8). Both control and experimental animals were fasted 24 hours. Following the fasting period 11 control rats received an intraperitoneal injection of 1 ml of corn oil. One ml of corn oil containing 0.2 ml of bromobenzene was administered to 15 experimental animals. Forty-eight hours later the rats were sacrificed by decapitation, livers were quickly removed, weighed on a Sartorius Balance and divided into 2 samples, one for histological examination and the other for lipide analysis.

The sample for lipide analysis from each liver was minced in an iced beaker and diluted 10-fold with cold 0.88 M sucrose. The mince was homogenized in a Potter-Elvehjem type tissue grinder with Teflon pestle for approximately 4 minutes in an ice bath, forced through a No. 20 gage hypodermic needle to remove stroma, and centrifuged in a Servall angle centrifuge (Type RR-1), according to a modification of the procedures of Hogeboom *et al.*(9) and Griffiths and Pace(10) for the separation of the cellular fractions by differential centrifugation. Mitochondria Fraction: An aliquot of the homogenate was centrifuged at 1,000 x g for 15 minutes to remove nuclei. The supernatant was then centrifuged at 10,000 x g for 30 minutes and the mitochondria precipitate was washed twice with cold 0.15 M NaCl and finally recentrifuged at 10,000 x g for 20 minutes. The mitochondria fraction was then treated with 10% trichloroacetic acid (TCA) containing 0.4 M $MgCl_2$(11) to remove the acid-soluble phosphorus and was then recentrifuged at 10,000 x g for 10 minutes. The precipitate was washed with 5% TCA containing 0.4 M Mg Cl_2 and recentrifuged at 10,000 x g for 15 minutes. The mitochondria precipitate was then covered with ethanol for dehydration for 6 hours and then washed quantitatively into Soxhlet extracting thimbles with ethanol. Nuclear Fraction: An aliquot of the homogenate was recentrifuged at 10,000 x g for 30 minutes. The precipitate was washed with cold 0.15 M NaCl and 10% and 5% TCA solutions and recentrifuged. The precipitate represents nuclei and mitochondria. The value of the nuclei component was obtained by difference. Homogenate Fraction: The homogenate fraction was precipitated by the addition of 10% TCA, centrifuged at 1000 x g for 15 minutes, washed with 5% TCA and recentrifuged. The lipides from the cellular fractions were extracted with 95% ethanol for 6 hours in Soxhlet continuous extractors. The lipides were purified with chloroform (12). Lipide P(13), cholesterol(14), lecithin and cephalin(15) were determined on aliquots of the chloroform solution. Histological examination showed that the mitochondria and nuclear fractions obtained corresponded in appearance and staining properties with those described by Claude(16) and Hogeboom(9).

Results. The lipide content of cellular fractions from the necrotic and control livers is reported in Table I. The *t* test of significance(17) was applied to the differences between the means for the control and experimental values to evaluate the statistical significance of the results. Histological examination of the liver section showed that necrosis was acute, central, fairly extensive and often confluent, extending between adjacent liver lobules in all specimens. The data in Table I show that bromobenzene necrosis produces a decrease in total lipides, lecithin and cephalin phosphorus in the homogenate fraction, the *t* test being 6.58, 3.90, 3.08 respectively. The probability, P, for chance occurrence of these differences was <0.01. There was a decrease in the lecithin phosphorus of the nuclei of the necrotic livers with a *t* value of 2.15 and P <0.05. The mean values of the total lipide, lecithin and cephalin phosphorus in the mitochondria fraction of the necrotic liver are lower. However, the difference is not significant. The data show that the cholesterol content was significantly greater in the necrotic liver nuclei and homogenate fractions. The mitochondria, nuclei and homogenate *t* values were 1.0, 5.30, 3.61 respectively. The probability, P, for chance occurrence of these differences was <0.01

TABLE I. Lipide Analysis of Cellular Fractions of Liver 48 Hours following Bromobenzene Administration.*

	mg of P or cholesterol per g of wet tissue											
	Mitochondria				Nuclei				Homogenates			
Exp.	Total lipide P	Lecithin P	Cephalin P	Cholesterol	Total lipide P	Lecithin P	Cephalin P	Cholesterol	Total lipide P	Lecithin P	Cephalin P	Cholesterol
Controls	.25 ± .04	.12 ± .02	.12 ± .02	.39 ± .09	.28 ± .05	.13 ± .05	.14 ± .03	.61 ± .11	1.07 ± .06	.53 ± .06	.46 ± .06	2.92 ± .26
Necrosis	.22 ± .03	.11 ± .02	.10 ± .02	.43 ± .09	.23 ± .04	.09 ± .02	.13 ± .03	1.26 ± .31	.85 ± .07	.42 ± .04	.35 ± .08	3.60 ± .48

* Figures preceded by ± sign indicate stand. dev.

for the nuclear and homogenate fractions. The rise of cholesterol content in the liver may be the result of increased synthesis, decreased catabolism or excretion. It is of interest that in necrotic liver of animals fed *Torula* yeast there has been observed a decreased conversion of labeled acetate to lipides(4). The pathogenesis of liver necrosis produced by *Torula* yeast is probably different from that of bromobenzene. However, the biochemical changes of lipides in liver necrosis produced by bromobenzene may reflect cellular activity and indicate that there is a lipide disturbance in this process.

Summary. (1) Lipide analyses of cellular fractions in liver necrosis produced by the administration of bromobenzene was studied in the rat. (2) A statistically significant decrease occurred in the total lipide, lecithin and cephalin phosphorus in the homogenate fraction of the necrotic livers as compared to the controls. However, the total cholesterol content was significantly increased. (3) In the nuclei of the necrotic livers a decrease in the concentration of the lecithin phosphorus and an increase of total cholesterol were noted.

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Oxidation of Unsaturated Fatty Acids in Embryonic and Adult Tissues of Golden Hamster.* (22735)

B. THEODORE COLE† (Introduced by F. G. Hall)
Department of Physiology and Pharmacology, School of Medicine, Duke University, Durham, N. C.

Kohn and Liversedge(1) gave evidence that rat tissue homogenates as well as slices incubated aerobically produced a compound which condensed with either p-amino benzoic acid (PAB) or thiobarbituric acid (TBA) and gave a pink color. Bernheim and co-workers showed that the color obtained by Kohn and Liversedge upon addition of TBA to incubated tissues was due to a product of the oxidation of certain unsaturated fatty acids (2). Crystalline hemoglobin and ascorbic acid were found to catalyze the reaction involved. Analysis of the TBA compound showed that a 3-carbon fragment, probably a peroxide, had combined with TBA. Such a reaction, namely, the oxidation of a double bond and the breaking of a fragment containing 3-carbon atoms from the end of the chain could occur in linolenic acid. Wilbur's group suggested the use of TBA as a test for oxidation of highly unsaturated fatty acids, principally linolenic(3). Donnan, working with rat tissues, noted that the results obtained in using the TBA test to estimate the amount of oxidized highly unsaturated fatty acid present in tissue preparations paralleled those obtained by the usual procedure for fat estimation(4). Holman, Lundberg and Malkin(5) reemphasized the TBA test as a means of qualitative and indirect measure of the oxidative products of highly unsaturated fatty acid (*i.e.*, linolenic, linoleic and oleic) content of tissues. Increased concentration of highly unsaturated fatty acids has been associated with increased rate of cell division(6). The following investigation was carried out to compare the oxidation of highly unsaturated fatty acids (HUFA) in terms of their oxidative products (HUFA-p) in brain, testes and liver, tissues of the embryo and young animal, with those of the adult. Experimental procedures were designed also to give evidence for *in vivo* oxidation of highly unsaturated fatty acids. Correlation between HUFA content and development of the tissues, as well as evidence of *in vivo* HUFA-p formation are presented.

Materials and methods. All pregnant females and weaned (15 days) animals derived HUFA's from a diet of Purina rabbit chow checkers supplemented with lettuce twice each week. The analysis of this diet, supplied by the Ralston Purina Co., is shown in Table I. About 50% of the 2.5% fat is made up of unsaturated fatty acids. Entire organs (with exception of adult livers, where only the left lobe was used) were removed immediately from decapitated animals, weighed wet, homogenized at 0-4°C and diluted with phosphate buffer, pH 6.0 to final concentration of 50 mg/ml. One 0.5 ml aliquot of this stock homogenate was added to 1.5 ml of buffer or to a mixture of 1 ml of buffer and 1.5 ml of ascorbic acid (concentration 0.2 mg/ml.) These mixtures contained in 50 ml Erlenmeyer flasks were either incubated in a con-

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† Department of Zoology, Physiology and Entomology, Louisiana State University, Baton Rouge.

TABLE I. Purina Rabbit Chow Checkers

Protein, %	15.00
Fat, %	2.50
Carbohydrate, %	55.00
Fiber, %	16.21
Ash, %	8.04
Calcium, %	1.12
Phosphorus, %	.65
Magnesium, %	.19
Iron, parts/million	161.00
Manganese, " "	55.00
Copper, " "	7.40
Cobalt, " "	.13
Iodine, " "	5.20
Potassium, %	1.03
Carotene (vit. A activity), parts/million	23.00
Thiamin, parts/million	3.70
Riboflavin, " "	7.40
Niacin, " "	38.80
Vit. D, U.S.P. units/g	2.21
Pantothenic acid, parts/million	6.62
Choline, " "	711.00

stant temperature bath for 2 hours at 37° or placed in cold centrifuge tubes and kept in ice bath for not longer than 30 minutes. The latter solutions so refrigerated were considered to represent a measure of the amount of oxidative product of the highly unsaturated fatty acid present in tissue *in vivo* at the time of removal. Values obtained from tissues incubated without ascorbic acid represent levels of HUFA oxidation as influenced by *in vivo* levels of vit. C, hemoglobin and perhaps other unknown protoplasmic catalysts. Total HUFA content of tissues is represented by TBA values for tissues incubated with ascorbic acid added *in vitro*. At end of incubation, or immediately, in the case of unincubated tissues, 1 ml of 20% trichloroacetic acid (TCA) was added and the precipitated protein was removed by centrifugation. To extract the chromogen precursor, at this step bound to the protein in the pellet, 2 ml of 5% TCA was added to the pellet. To the supernatant, and to tubes containing protein and 5% TCA, was added 2 ml of 0.75% thiobarbituric acid. These mixtures were placed in boiling water bath for 15 minutes. All tubes were subsequently returned to total volume of 5 ml with distilled water and the color read and recorded at wave length 525 on Coleman Junior Spectrophotometer. The optical density of supernatant was expressed in terms of Q values read directly from Spectrophotometer. These values were interpreted as a qualitative expression of the fraction of HUFA-p unbound, and values representing color extracted from the pellet were interpreted as a measure of the HUFA-p bound to protein. All values were read against a blank of 2 ml of 20% TCA, 2 ml of 0.75% TBA and 1 ml distilled water. All studies were carried out in duplicate. Values shown in Tables and Figures are in terms of color density produced by 25 mg of tissue and represent total color produced (bound plus unbound fractions).

Results are presented in Figs. 1, 2, and 3. Data on tissues from embryos 14-15 days old are plotted above abbreviation "Emb" and those for tissues from animals within 8 hours *post partum* above the letters "JB". Incubated tissues at every age gave considerably higher values than unincubated tissues. It is also evident that the catalytic action of ascorbic acid is manifest only in incubated tissues. Statistical analysis of unincubated liver and testes tissue data shows no significant difference between values obtained for different aged animals. In brain tissue, however, the values are significantly higher

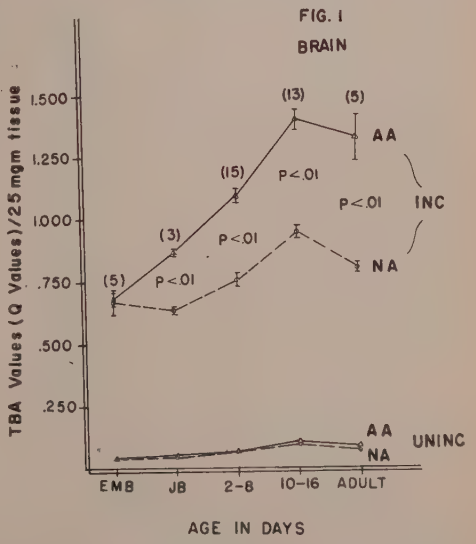


FIG. 1. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in brain tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

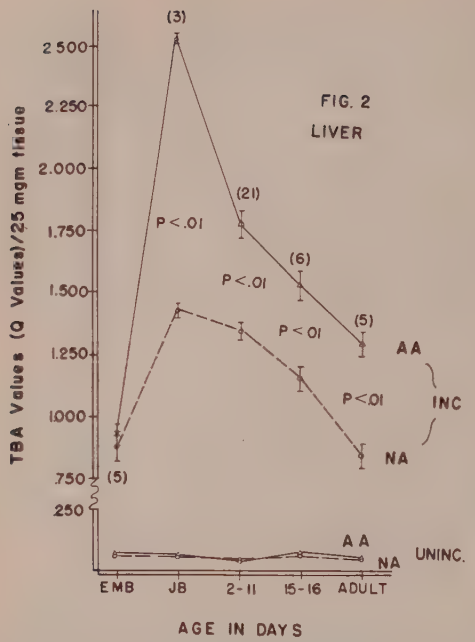


FIG. 2. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in liver tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

($P < .01$) in animals 10 days of age and older, than in embryos or in tissues of animals at birth. Brain values of animals above 10 days old are higher than either liver or testes values, indicating the presence of greater quantities of HUFA oxidized *in vivo* in this tissue.

TBA values for brain tissue incubated with ascorbic acid show significant increases from embryonic tissues through 10 days of age (Fig. 1). Values for tissues of embryo and those of animals through 8 days of age, incubated without ascorbic acid, are significantly lower than values for brain tissue of animals beyond 10 days of age. Catalytic action of ascorbic acid on oxidation of HUFA in embryonic tissue is not apparent.

TBA values for liver tissue incubated with ascorbic acid show a pattern in reverse to that of the brain (Fig. 3 and 4). Although oxidation of HUFA in embryonic liver tissues as in brain, does not reflect the catalytic action of vit. C, values for tissues incubated with ascorbic acid are extremely high at birth and thereafter show a gradual and significant decline to adult levels. The latter are substantially elevated above values for embryonic tissue. Tissues incubated without ascorbic acid indicate a similar trend except that levels in the adult are not significantly different from those in the embryo.

Values for testes tissue incubated with ascorbic acid show an increase from a mean level of .701 in animals 15-30 days of age to a peak of 1.331 in animals 55-65 days of age (Fig. 3). Tissues incubated without ascorbic acid reflect a similar trend with peak in group 35-65 days old.

Discussion. Consideration of TBA values for unincubated liver, brain and testes tissues give evidence of a measurable amount of HUFA oxidized *in vivo* with oxidative products present in tissue at time of sacrifice. These conclusions are based upon the fact that measurable quantities of HUFA-p were found in tissues maintained at low temperature and in consideration of the fact that if oxidation occurred *in vitro* it appears to be a valid assumption that it would be catalyzed by the ascorbic acid added. Bernheim, Wilbur and Kenaston(7) have suggested *in vivo* formation of oxidized fatty acid products may act as normal regulators of some aspects of

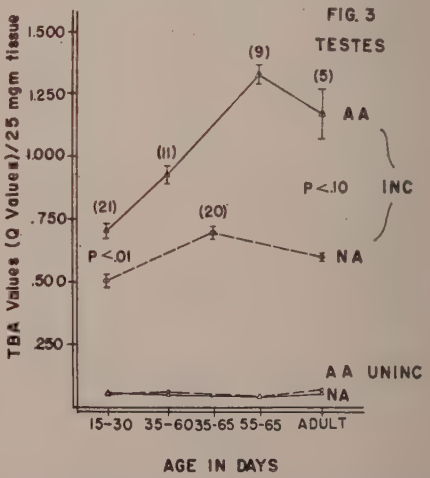


FIG. 3. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in testes tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

metabolism. Wilbur and co-workers(8) suggest a role of HUFA-p in inhibition of cell division. The oxidative product may well be labile *in vivo* and the values here defined represent those "stabilized" by death of animal and its constituent protoplasm. Except in brain tissue of animals above 10 days old, there is no significant correlation between change of *in vivo* levels of HUFA-p and age. Variation in TBA values of tissues incubated without ascorbic acid reflect *in vitro* oxidation of HUFA in presence of unknown concentration of ascorbic acid which is a constituent part of the tissue under investigation. Therefore, variation in these TBA values may be interpreted as evidence for variation in ascorbic acid content of tissues of animals at various ages, rather than a quantitative expression of HUFA present. Values for tissues incubated with ascorbic acid, under these experimental conditions, probably give a qualitative estimate of total highly unsaturated fatty acid content of the tissue studied.

In the case of brain tissue the content of HUFA gradually rises to that of adult levels in the 10-day-old animal. Since neither the adult nor the embryo is known to synthesize highly unsaturated fatty acids, and in light of the work of Bickenback and Rupp(9) it can be assumed that the only source of HUFA to the embryo is via maternal circulation(10, 11,12). It is significant to note that TBA values in the animals here studied are established at adult levels when the animal is weaned and begins to eat "hard" food, 10-16 days *post partum*. Values for tissues incubated without ascorbic acid do not rise appreciably in the adult, which fact eliminates the probability of an inhibitory action of ascorbic acid added *in vitro*. The abrupt rise in HUFA content of liver tissues correlates well with alteration in liver function known to occur at birth. The findings may be exaggerated by catalytic action of hematopoietic components in the liver during this interval. The gradual rise in total HUFA content of testes tissue from 15 days to a peak in the 55-56 age bracket suggests a significant relationship between fatty acid content, development, and function of the organ. The peak in HUFA content correlates well with age of sexual maturity in the hamster. The gradual, increased difference in TBA values correlated with age, between tissues incubated with and without ascorbic acid suggests a gradual alteration in the *in vivo* content of ascorbic acid.

Perhaps the most significant finding is lack of evidence for catalytic activity of vit. C on *in vitro* oxidation of HUFA constituents of embryonic tissues. This could be due to inhibitory effects of high concentration of ascorbic acid (resulting from high *in vivo* levels), or could be the result of an inhibitory substance peculiar to embryonic tissues and not found in tissues *post partum*. The possible role of variable tissue concentrations of tocopherols and other metabolic antioxidants should be mentioned. Although no bio-assay of vit. E was made, the diet used is sufficient in every respect to sustain our colonies of hamsters indefinitely without showing symptoms of vitamin deficiency. The possibility exists that where TBA values for tissues incubated without ascorbic acid are low, tissues themselves may contain high concentrations of antioxidants not readily identified. Where no difference can be demonstrated between levels of HUFA-p in tissues incubated with and without ascorbic acid (*i.e.*: embryonic tissues) a reaction between some unidentified antioxidant may be manifest. Ability of specific tissues of various aged animals to concentrate tocopherol remains a possibility. Current investigation of this problem is being undertaken.

Summary. The Thiobarbituric Acid Test for oxidative products of essential fatty acids (HUFA), primarily linolenic, present in tissues *in vivo* and after aerobic oxidation *in vitro,* with and without ascorbic acid, was applied to brain, liver and testes tissues of hamsters of various ages. Minute measurable quantities of oxidative products (probably peroxides) are formed *in vivo*. The quantity of HUFA present in brain tissue increased gradually with *post partum* development of the brain, with constant levels apparent after 10-16 days of age. The quantity of HUFA present in testes tissue correlates well with development and function of that organ

with constant levels established, following a peak during age of sexual maturity. There is a significant rise in total HUFA content of liver tissue, associated with emergency of birth. Lack of catalytic activity of ascorbic acid upon *in vitro* oxidative reaction involved in embryonic tissue has been demonstrated.

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Influence on Circulating 17-Hydroxycorticosteroid Concentrations of Compounds Structurally Related to Salicylate.* (22736)

ALAN K. DONE,† ROBERT S. ELY, AND VINCENT C. KELLEY

Department of Pediatrics, University of Utah College of Medicine, Salt Lake City

Earlier publications(1,2) reported the finding of marked elevations in circulating 17-hydroxycorticosteroid (17-OHCS) concentrations in guinea pigs following intraperitoneal or oral administration of large doses of sodium salicylate. Hypophysectomy, as well as adrenalectomy, abolished this effect(2). In the present investigation, various other derivatives of benzoic acid were studied with regard to their effects on circulating 17-OHCS concentrations in guinea pigs in an attempt to ascertain whether these effects are related to the structural configuration of the administered compound.

Methods. Male guinea pigs weighing 450 to 750 g were used in these experiments. Sodium salts‡ of the compounds listed in Table I were prepared by dissolving the acids in an equivalent amount of 1 N NaOH; the pH of each solution then was adjusted to neutrality using the Beckman glass electrode. Solutions were diluted with distilled water to contain the equivalent of 100 mg of the acid per ml. Compounds tested were injected intraperitoneally in doses equivalent (on the basis of molecular weight) to 200 mg salicylic acid per kilo body weight. Four hours later the animals were rapidly anesthetized in an ethyl ether atmosphere and bled from the abdominal aorta into heparinized syringes. Additional groups of animals treated with the monohydroxy derivatives of benzoic acid were sacrificed at 1 and 2 hours following injection. Plasmas were separated by centrifugation immediately and stored in deep-freeze until used for analyses. Plasma concentrations of 17-OHCS were determined by the method of Nelson and Samuels(3) as modified by Eik-Nes, Nelson and Samuels(4).

Observations. Data concerning the influence on plasma 17-OHCS concentrations of the various compounds tested are shown in Table I. Benzoic acid had no significant effect. For purposes of statistical analysis the

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‡ For convenience, compounds will be referred to as acids.

TABLE I. Influence of Benzoic Acid Derivatives on Circulating 17-OHCS Concentrations in Guinea Pigs. (Doses equivalent to 200 mg/kg salicylic acid.)

Compound†	No. determinations	17-OHCS* (μg/100 ml)	P vs. benzoic acid	P vs. salicylic acid
Untreated	51	37 ± 2.7‡		
Benzoic acid	4	43 ± 5.2		
Monohydroxy derivatives:				
o-OH (salicylic)	5	81 ± 5.6	<.01	
m-OH[1,2]	4	58 ± 9.3	>.05	>.05
p-OH[1,2]	5	57 ± 3.0	>.05	.01–.02
Ether derivatives:				
o-Methoxy[1,2]	7	76 ± 6.7	<.01	
o-Ethoxy[2]	4	119 ± 11.2	<.01	.02
m-Methoxy[2,3]	5	75 ± 11.3	.02–.05	
p-Methoxy[1,2]	5	77 ± 16.7	>.05	
p-Ethoxy[1,2,4]	3	50 ± 2.4	>.05	<.01
3,4,5-Trimethoxy[2,4]	4	119 ± 11.1	<.01	.02
Amino derivatives:				
o-Amino[5]	5	62 ± 4.7	.02–.05	.02–.05
p-Amino (PABA)	3	71 ± 6.9	.02–.05	
Dihydroxy derivatives:				
2,3-OH (o-pyrocatechuic)[4]	3	127 ± 16.6	<.01	.02–.05
2,4-OH (β-resorcylic)[1–4]	9	89 ± 18.6	.02–.05	
2,5-OH (gentisic)[1,4,6]	7	86 ± 11.5	<.01	
2,6-OH (γ-resorcylic)[1,2]	5	123 ± 10.2	<.01	<.01
3,4-OH (protocatechuic)[3]	2	(26, 67)		
3,5-OH (α-resorcylic)[1,4]	4	81 ± 11.6	.02–.05	
Mixed derivatives:				
2-OH, 3-Methyl[1,2,4]	2	(132, 77)		
2-OH, 4-Methyl[2,4]	4	117 ± 14.2	<.01	.05
2-OH, 3-Methoxy[3,6]	7	110 ± 6.8	<.01	<.01
2-OH, 5-Methoxy[2,4]	7	117 ± 18.9	<.01	>.05
2-OH, 4-Amino (PAS)[2]	5	64 ± 16.1	>.05	>.05
Acetylsalicylic acid	7	132 ± 15.2	<.01	.01
Salicyluric acid[2]	4	36 ± 5.9		<.01

* Four hr after intraper. inj. † Each administered as the sodium salt. ‡ Mean ± S.E.

[1] Kindly supplied by Eli Lilly & Co., Indianapolis, Ind.
[2] " " " S. E. Massengill Co., Bristol, Tenn.
[3] " " " The Squibb Institute for Medical Research, New Brunswick, N. J.
[4] " " " Parke-Davis & Co., Detroit, Mich.
[5] " " " Dow Chemical Co., Midland, Mich.
[6] " " " Abbott Laboratories, North Chicago, Ill.

group of animals given benzoic acid were considered the control group. The reasons for this were that the number of animals corresponded more closely with those in the other experimental groups and the animals were treated similarly in that they received an intraperitoneal injection of a potentially irritating solution which in each case contained an equivalent amount of sodium and was of identical pH. *Monohydroxy derivatives of benzoic acid.* The animals given o-hydroxybenzoic (salicylic) acid had highly significant elevations of plasma 17-OHCS concentrations. In contrast, the groups given m- or p-hydroxybenzoic acid did not have significant elevations of 17-OHCS concentrations at the 4 hour interval. *Ether derivatives of benzoic acid.* The o-substituted methoxy and ethoxy derivatives likewise were active in producing elevated 17-OHCS concentrations, the latter appearing perhaps to be more active than either the o-methoxy compound or salicylic acid. The elevations of plasma 17-OHCS produced by the m- and p-methoxy derivatives are of questionable significance because of wide variations in values among the animals tested; however, no significant effect was observed with p-ethoxybenzoic acid. On the other hand, 3,4,5-trimethoxybenzoic acid produced highly significant elevations of

plasma 17-OHCS concentrations, possibly greater than those produced by salicylic acid ($p = .02$). *Amino derivatives of benzoic acid.* Two mono-amino benzoic acids, the o- and p- derivatives, were studied. Both of these compounds produced steroid elevations of borderline significance ($.02 < p < .05$ vs. benzoic acid). *Dihydroxy derivatives of benzoic acid.* Among the dihydroxybenzoic acids, the 2,3; 2,5 and 2,6-substituted derivatives resulted in highly significant plasma 17-OHCS elevations. Elevations of borderline significance ($.02 < p < .05$ vs. benzoic acid) occurred with the 2,4 and 3,5-substituted compounds. Insufficient data are available for the 3,4-substituted compound to permit conclusions. *Mixed derivatives of benzoic acid.* Derivatives of o-hydroxybenzoic (salicylic) acid with substitution of a methyl group at the 3 or 4 position or a methoxy group at the 3 or 5 position produced elevations of plasma 17-OHCS concentrations which were highly significant. With the small numbers of animals studied there appeared to be no significant difference between p-aminosalicylic acid and benzoic acid in their effects on 17-OHCS concentrations.

The greatest mean elevation of plasma 17-OHCS concentrations in any group of animals occurred in that group given acetylsalicylic acid (aspirin), the response being significantly greater than that obtained with salicylic acid. Salicyluric acid exerted no effect on plasma 17-OHCS concentrations. *Time relationship of steroid response to monohydroxybenzoic acids.* Table II concerns plasma 17-OHCS concentrations at varying intervals following treatment with the monohydroxybenzoic acids. Animals treated with o-hydroxybenzoic (salicylic) acid had no significant elevation of plasma 17-OHCS concentrations at 2 hours, but had highly significant elevations at 4 hours. Those treated with m-hydroxybenzoic acid showed a suggestive ($.02 < p < .05$ vs. benzoic acid) elevation of 17-OHCS concentrations at 2 hours. p-Hydroxybenzoic acid did not significantly affect 17-OHCS concentrations at any of the 3 sampling intervals (1, 2 or 4 hours).

TABLE II. Plasma 17-OHCS in Guinea Pigs at Varying Intervals following Treatment with Monohydroxy-Benzoic Acids. (Doses, 200 mg/kg by intraperitoneal injection.)

Compound*	Sampling interval 1-hr	2-hr	4-hr
o-Hydroxybenzoic (salicylic) acid		44 ± 10.9 (3)	81 ± 5.6 (5)
m-Hydroxybenzoic acid	63 ± 7.3† (3)	71 ± 8.5 (4)	58 ± 9.3 (4)
p-Hydroxybenzoic acid	49 ± 8.8 (4)	58 ± 6.6 (2)	57 ± 3.0 (5)

* Each administered as the sodium salt.
† Mean ± S.E.
No. in parentheses = No. of determinations.

Discussion. Equimolar doses of each of the compounds studied were administered in order to permit estimations of relative potency of these compounds in producing elevations of circulating 17-OHCS concentrations. However, certain variables preclude strict comparisons. Substantial differences may exist, among the compounds tested, in absorbability from the peritoneal cavity. The sampling interval of 4 hours was chosen because the maximum steroid response to salicylate was found to occur at that time(1). However, maxima for the other benzoic acid derivatives may not have coincided with the time of sampling used in these studies. Moreover, although the dose of each compound was equivalent on a molar basis there was wide variance in the ratio of administered to toxic dose. A non-specific toxic effect therefore is more likely with some of these substances than with others. None of the compounds studied was lethal in the dose employed. The majority of the compounds used in the present investigation have been studied only to a limited degree, or not at all, with respect to antiphlogistic potency. Moreover, where such data are available, categorical conclusions as to whether or not the particular compound has antiphlogistic properties cannot be drawn in most instances since results have differed from one type of experiment to another. Therefore, any comparison of this effect with influence on 17-OHCS concentrations necessarily must be incomplete and will be attempted only insofar as available data permit.

Among the monohydroxy derivatives of

benzoic acid, only the o-substituted compound (salicylic acid) exerted a pronounced effect on 17-OHCS concentrations at the 4 hour post-injection interval. Blanchard, *et al.* (5) and Cronheim, *et al.*(6) found the m-compound to have no effect on adrenal ascorbic acid, but others(7-10) have found that it produces significant depletion. Smith(9) reported significant adrenal ascorbic acid depletion with both m- and p-hydroxybenzoate. However, the doses employed (330 and 400 mg/kg, respectively) were much higher than those used in the present study and produced less depletion of adrenal ascorbic acid than did comparable doses of salicylate or even benzoate. The author attributed the differences in response to the lower circulating concentrations of the m- and p-hydroxybenzoates. He found that the peak plasma concentration of p-hydroxybenzoate occurred 30 minutes after intraperitoneal injection; none could be detected 1½ hours later. The greatest depletion of adrenal ascorbic acid was noted after the former, and no significant depletion after the latter, interval. This finding was suggestive that the interval between injection and removal of the adrenals may have been too long in the experiments of other workers(5,6) who failed to demonstrate an effect of this compound on adrenal ascorbic acid content. The failure in the present study to demonstrate a significant effect on circulating 17-OHCS at a time when the salicylate effect is maximum (4 hours after injection) might be explained similarly. For this reason, further studies of animals treated with m- and p-hydroxybenzoic acid were made at one- and 2-hour intervals. In the case of p-hydroxybenzoic acid, no effect on 17-OHCS concentrations was demonstrable. With m-hydroxybenzoic acid there was a mean 17-OHCS concentration at 2 hours which was elevated by comparison with benzoic acid-treated ($.02<p<.05$), or untreated ($p<.01$), animals; no significant effect was demonstrable at 4 hours following injection. Of the 3 monohydroxy derivatives of benzoic acid, only o-hydroxybenzoic (salicylic) acid has been reported to have antirheumatic properties(11) and to inhibit the development of "anaphylactic arthritis"(12).

The effects of certain other derivatives of benzoic acid on circulating 17-OHCS concentrations appear not to parallel their effects on adrenal ascorbic acid concentrations. Although o-methoxy and o-ethoxybenzoic acid both caused definite elevations of 17-OHCS concentrations, these compounds have been found not to deplete adrenal ascorbic acid (6,7). Plasma 17-OHCS concentrations following m- or p-methoxybenzoic acid were so variable that although the mean 17-OHCS values in these groups were comparable with those of salicylate-treated animals, these elevations were not statistically significant with the small numbers of animals used. Of these compounds, only p-methoxybenzoic acid has been found to deplete adrenal ascorbic acid significantly(7). p-Ethoxybenzoate has no effect either on 17-OHCS concentrations or adrenal ascorbic acid(6).

Administration of the o- and p-amino derivatives of benzoic acid resulted in mean 17-OHCS concentrations which were intermediate between those produced by benzoic acid and salicylate. p-Aminobenzoic acid has been reported to have no effect on adrenal ascorbic acid concentration(6) and to have no(13), or suggestive(14), antirheumatic properties. Neither compound was reported to inhibit anaphylaxis(15).

The addition of a second hydroxyl or an ether group to the salicylate structure appeared, in certain instances, to enhance the effect on circulating 17-OHCS. Thus, treatment with 2,3-dihydroxybenzoic acid resulted in suggestively, ($.02<p<.05$), and 2-hydroxy, 3-methoxy- or 2,6-dihydroxy-benzoic acid in definitely ($p<.01$), greater elevations of 17-OHCS concentrations than did salicylic acid. 2,6-Dihydroxy benzoate (γ-resorcylate) has considerably greater antirheumatic potency than salicylate(16). Gentisic acid had essentially the same magnitude of effect on 17-OHCS concentrations as salicylic acid and has been reported to have comparable antirheumatic potency, despite observations that it produces a somewhat lesser depletion of adrenal ascorbic acid(6,7,10).

A somewhat surprising finding was the sig-

nificantly greater effect on circulating 17-OHCS concentrations of aspirin than of salicylic acid when the 2 compounds were given in equimolar amounts. Indeed, aspirin produced the greatest effect on 17-OHCS concentrations of any of the compounds tested. The increment in steroid response to aspirin over salicylic acid is proportional to the increment in milligram dose, suggesting that the 2 compounds may have similar potency when administered in equal quantities.

Salicyluric acid, the principal metabolite of salicylate, exerted no effect on 17-OHCS concentrations. This substance has been reported to cause a questionable depletion ($p = .02$) of adrenal ascorbic acid(6) but no inhibitory effect on "anaphylactic arthritis" (12).

Since relatively few of the compounds investigated in the present study have been evaluated with regard to antirheumatic potency, it is difficult to assess the relationship of this property to ability to produce elevated circulating corticosteroid concentrations. However, insofar as available data permit such comparison, these two effects appear to correlate well. On the other hand, there appear to be numerous discrepancies between adrenal ascorbic acid-depleting ability of these drugs and either their antirheumatic effects or their influence on plasma 17-OHCS concentrations.

Summary. 1. Twenty-five compounds representing structural derivatives of benzoic acid were studied with regard to their effects on circulating 17-hydroxycorticosteroid (17-OHCS) concentrations in guinea pigs. 2. Benzoic acid had no effect on these concentrations. 3. Among the monohydroxybenzoic acids, the o- and m-substituted derivatives produced elevated plasma 17-OHCS concentrations. The latter compound exerted its effect earlier following administration than did salicylate. p-Hydroxybenzoate had no significant effect. 4. Among the mono-ether derivatives of benzoic acid only the o-substituted compounds produced significant elevations of plasma 17-OHCS concentrations. Significant elevations were produced by 3,4, 5-trimethoxybenzoic acid. 5. Both o- and p-aminobenzoic acid produced steroid elevations of borderline significance. 6. The addition to the salicylate structure of a second hydroxyl group in the 3 or 6 position or of an ether group in the 3 position appeared to augment the effect on 17-OHCS concentrations. 7. Aspirin exerted the greatest effect on plasma 17-OHCS concentrations of any compound tested. 8. The salicylate metabolite, salicyluric acid, had no effect. 9. A comparison of the influence of these compounds on 17-OHCS concentrations with antirheumatic and anti-inflammatory properties and with effects on adrenal ascorbic acid concentrations, as reported by others, was attempted.

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Errors in Plasma Volume Measurement from Adsorption Losses of Albumin-I^{131}.* (22737)

E. B. Reeve and J. J. Franks†
Department of Medicine, University of Colorado Medical Center, Denver

Recently, when using albumin-I^{131} to measure plasma volume, we obtained unexpectedly low results. Adsorption losses of the albumin-I^{131} to the walls of containers offered a possible explanation, and experiments showed this to occur. The degree of error introduced in plasma volume measurement by this adsorption and methods of preventing it have been studied.

Methods. Various samples of a commercial preparation of human albumin-I^{131},‡ containing 50 to 140 μC I^{131} per mg albumin when received, were used. Ninety-six to 98% of the radioactivity was protein-bound as shown by precipitation with 5% trichloroacetic acid. When received the material containing 1 mC was diluted in 30 ml sterile 0.9% NaCl. This stock solution was stored in the refrigerator and discarded after 3 to 4 weeks. Radioactivity was measured in capped vials in a well scintillation counter. All glassware was cleaned in concentrated chromic acid-sulfuric acid cleaning mixture (100 g K dichromate in 200 ml H_2O, treated with 1000 ml conc. H_2SO_4) for from 0.5 to 24 hours, then rinsed repeatedly with hot water, cold water and distilled water. Of the many solvents tested only chromic acid-sulfuric acid completely removed the adsorbed radioactivity from glass in a few minutes at 25°C. Hence this mixture was used for "extracting" the bound radioactivity. The loss by adsorption to pipettes and containers made special precautions necessary for the accurate preparation and assay of dilute solutions of albumin-I^{131}. Losses proved negligible in a micrometer syringe filled with a solution containing 200 or more μg protein/ml, and more dilute solutions were therefore prepared by delivering a minute volume of a relatively concentrated solution from this syringe into the solvent. To prevent losses in 1 ml transfer pipettes, these were first filled and emptied thrice with a 1 mg/ml solution of plasma albumin or dilute human plasma. Then air was sucked through them till the lower 3 inches of the pipette was almost dry. After this treatment, at most 2% of the radioactive contents was adsorbed.

Results. Table I summarizes the results of a typical experiment in which 27 μg of albumin-I^{131} were transferred to 4 pairs of 50 ml and 4 pairs of 250 ml flasks. To each pair was added one of the 4 solvents—distilled water, 0.9% NaCl, 0.1 N NaOH and 0.9% NaCl containing 1 ml human plasma/100 ml. The flasks were mixed by repeated inversion initially and before removing the samples. It is seen that counts are lost from water and sodium chloride solution and that these losses increase with storage at low temperature overnight and are proportionately greater from the more dilute solutions. Most of the lost counts are regained within a few minutes after adding NaOH. Counts are not lost from stored solutions which contain the carrier protein. A number of similar experiments showed: a) that KI in 1% concentration or less did not prevent loss of counts from water solutions; b) 3 and 5% NaCl, 1 and 3% $NaHCO_3$, and 3% Na Acetate did not prevent loss of counts; c) irregular small losses of counts occurred from 0.1 N NaOH and 1% Na_2CO_3 solutions; d) the 1% detergent solutions used by Sear *et al.*(6)§ completely blocked the loss of counts for several days at 4°C; e) addition of sufficient human serum albumin or human plasma, with or without KI, blocked the loss of counts from 0.9%

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† At present on duty with the U. S. Air Force (M.C.).

‡ From Abbott Laboratories, N. Chicago, Ill.

§ One percent Alconox cleaning solution.

TABLE I. Loss of Counts from Albumin-I^{131} Solutions in Volumetric Flasks.

	Distilled H_2O	.9% NaCl	.1 N NaOH	Carrier protein in .9% NaCl
A. 27 μg Albumin-I^{131} in 50 ml of solvent				
Counts/min./ml				
After 90 min. at 25°C	18240 (85%)	19160 (89 %)	21740 (101 %)	21560 (100 %)
After 20 hr at 4°C	13700 (64%)	17100 (80 %)	21000 (97.5%)	21360 (99 %)
B. 27 μg Albumin-I^{131} in 250 ml of solvent				
Counts/min./ml				
After 90 min. at 25°C	3190 (75%)	3760 (88.5%)	4390 (103 %)	4250 (100 %)
After 20 hr at 4°C	1960 (46%)	3050 (72 %)	4160 (98 %)	4350 (102.5%)
After adding 300 mg NaOH	4160 (98%)	4040 (95 %)		

NaCl completely for as long as 2 weeks at 4°C.

Figure 1 shows the results of 2 of a number of experiments designed to measure the adsorption of protein to glass from 0.9% NaCl solution at room temperature. In Exp. A, 3 ml protein solution was exposed to the inner surface of 10 × 75 mm Pyrex test tubes capped with Parafilm and mixed by inversion initially and at 10 minute intervals thereafter; in Exp. B, 15 ml protein solution was exposed to the inner surface of 15 × 125 mm Pyrex test tubes with mixing only initially. At concentrations of protein of the order of 1 μg/ml or less, 10% to 50% of the protein is adsorbed to the walls of the test tubes. As the protein concentration increases the proportion of the total adsorbed decreases, until at concentrations of between 10 and 30 μg/ml, only 2 to 5% of the total is adsorbed. In Fig. 2 the μg protein adsorbed per cm^2 glass in Experiments A and B are plotted against the *final* concentrations of protein in solution. (The *final* concentrations were obtained by subtracting the quantity adsorbed from the quantity added initially to the test tube). The quantity of protein adsorbed by unit area of surface increases over the whole range of concentrations studied, but most rapidly over the range from 0.01 to 5 μg/ml (Fig. 2 insert). A few experiments showed that when 3 ml solution of 2 μg albumin-I^{131}/ml saline is mixed by repeated inversion in 10 × 75 mm test tubes, one half the equilibrium value is adsorbed in from 1 to 5 minutes and equilibrium appears to be reached in from 5 to 20 minutes. Albumin-I^{131} adsorbs to surfaces other than glass. It adsorbs vigorously to polyethylene, as noted by Sear, Allen and Gregersen(6). We have observed that albumin-I^{131} also adsorbs to celluloid and other transparent plastics, to glass tubes coated with silicone and histological paraffin, and to stainless steel and rubber.

Discussion. Different samples of human albumin-I^{131} show some variation in adsorption but in general the adsorption isotherms

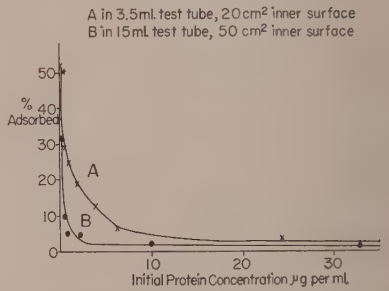


FIG. 1. % of total albumin-I^{131} adsorbed in 1 hr plotted against initial conc. (μg/ml).

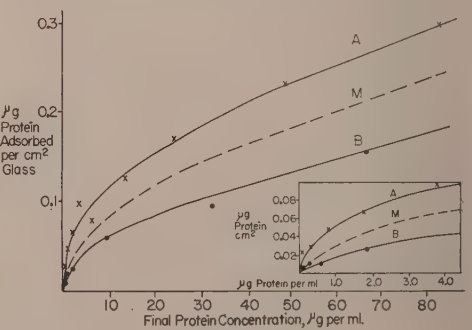


FIG. 2. Relation between protein conc. and quantity of protein adsorbed to glass.

lie in the area bounded by curves A and B, Fig. 2. We find also that rabbit albumin-I^{131} and human gamma globulin-I^{131} adsorb similarly. Recently Bull(2) has reported quantities of unlabelled bovine albumin adsorbed to glass from buffer of pH 4.66 that would fit well with a projection of curve A, Fig. 2. The errors due to adsorption in a plasma volume measurement depend on the fraction of the total albumin-I^{131} adsorbed to the glassware. This depends on the solvent, protein concentration, area of surface, time of exposure to surface, temperature and the effectiveness of mixing in the solution. At room temperature with 0.9% NaCl as solvent the approximate errors resulting from adsorption can be calculated from Curve M, the mean of curves A and B, Fig. 2, provided that the surface area of the glassware and the protein concentrations of the solutions are known. Calculations show that the surface volume relationships are of great importance. Thus, volumetric glassware with a surface of less than 1 cm^2 per ml contained solution adsorbs less than 1% of the contained protein at concentrations of 10 μg/ml, and less than 5% at concentrations of 1 μg/ml, whereas, glassware with a surface of 20 cm^2 per ml may adsorb 15% of the contained protein at 10 μg protein/ml, and 35% at 1 μg/ml. Examples of the former glassware are volumetric flasks of 100 ml capacity or greater, and of the latter, 1 ml Tuberculin syringes and straight 1 ml pipettes. (Pipettes would be a very grave source of error were it not that they are filled and emptied rapidly, and, since albumin diffuses slowly, time is insufficient for equilibrium to be reached. Experiment shows that with each filling a few per cent of the contents is adsorbed until the surface becomes saturated.) The calculated percentages of protein that become adsorbed during a plasma volume measurement, in which 10 ml of 0.9% NaCl solution of albumin-I^{131} containing 10 to 40 μg protein/ml are injected, and the radioactivity in 1 ml of plasma separated from the blood samples is compared with that of 1 ml of a 1 in 100 dilution of the injection solution are: In the syringe, 3 to 6; in the glassware containing the separated plasma, nil; in the glassware used for the standards (a) 100 ml volumetric flask, 8 to 10; (b) two 1 ml transfer pipettes, 2 to 3 each. Since losses in the syringe lead to an overestimate whereas those occurring during the preparation of the standards lead to an underestimate of plasma volume, these errors partially cancel out. As a result, plasma volume is underestimated by 8 to 12% depending on the protein concentration of the injected solution. In unfavorable circumstances such as storage of standards in the cold and preparation of standards with distilled water, greater errors would arise. Schultz *et al.*(5) who measured radio activity with G-M counters, added plasma to their standards to equalize adsorption losses of B rays; Sear *et al.*(6) prepared their standards in 1% detergent. These two groups of workers showed good agreement between plasma volume measurements made in man and dog with T1824 and albumin-I^{131}. Other workers (*e.g.*1,7) have failed to find such good agreement. Recently, technics using scintillation counters have been described(3) in which losses by adsorption would be anticipated; Gamble *et al.*(4) have already noted such losses. It is clear that to make precise measurements these losses must be avoided. This may be achieved as follows: When the albumin-I^{131} is prepared for injection at least 1 mg carrier protein per ml solution is added. For carrier protein either the patient's plasma or a preparation of human plasma albumin is used. To make the standards a measured volume of the solution prepared for injection is diluted in a volumetric flask, either with 0.9% NaCl containing 0.5 mg plasma protein/ml, or with 1% detergent solution.

Summary. Adsorption losses of albumin-I^{131} to volumetric glassware may be sufficient to cause significant underestimates of plasma volume. Some factors affecting these losses are examined and methods of minimizing their effects are described.

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Erythropoietic Stimulating Effects of Plasma Extracts from Anemic Human Subjects. (22738)

SAM J. PILIERO, PAUL T. MEDICI, BEN PANSKY, A. LEONARD LUHBY, AND ALBERT S. GORDON

Departments of Anatomy and Pediatrics, New York Medical College, Flower-Fifth Avenue Hospitals, and Departments of Biology, Graduate Schools of Arts and Science, St. John's University, Brooklyn, N. Y. and New York University, New York City

It is now a well documented finding that various types of anoxia evoke the production, in laboratory animals, of circulatory factors capable of stimulating erythropoiesis(1-11). These observations raise the question as to whether such activity is also demonstrable in the blood of patients with various forms of anemia. Few studies have attempted to assess the erythropoietic (EP) properties of body fluids of human subjects. Some of the experiments have been performed on umbilical cord blood of normal newborn babies and have indicated EP activity in such material when tested in rodents and man(12-16). EP activity is also stated to be present in the serum of a man sojourning at altitude for 8 days (12), in the plasma of patients with chronic heart disease(1), in human subjects with anemia following hemorrhage(17) and in infants with erythroblastosis fetalis(16). Plasma from subjects with congenital spherocytosis or pernicious anemia in reticulocyte crisis was ineffective(18). These studies with human material fail to evaluate the response of the blood-forming organs in the test animals. The criteria used by the investigators, namely red cell (RBC) counts and/or reticulocyte response, cannot always be depended upon as reflecting the degree of EP stimulation(19,20).

The present studies are concerned with the effects exerted by extracts of plasma and one of urine from patients with certain types of anemia. Since previous experiments(5-8,10) indicate that more of the EP factor is recoverable in hemolytic than in other types of experimentally induced anemias, possibly because the hemolytic varieties are usually more severe, it was felt that an examination of the effects of body fluids secured from patients with Cooley's and sickle cell anemia might be rewarding.

Materials and methods. Plasma was obtained from 7 authenticated cases of Cooley's anemia (6 children and 1 adult) and 2 patients with sickle cell anemia. One case of chronic hypoplastic anemia in a child is also included. Four normal subjects served as donors for control plasma. A 24 hour sample of urine from one of the Cooley's subjects was also tested. In Table I are indicated the hemoglobin concentration, reticulocyte percentage and nucleated RBC numbers in the peripheral blood of each of these individuals at the time of blood withdrawal.

Because of the effectiveness of an acidification-boiling procedure in revealing the EP factor in the blood and urine of anoxic rodents (5-7,10,11,21), it was decided to utilize this extraction technic for the plasmas of the anemic patients. Blood collected from each of the patients was heparinized (10 mg/50 ml blood). The plasmas were acidified to pH 5.5 with lN HCl and boiled for 10 minutes. Following filtration, the coagulated residues were discarded and the filtrates neu-

TABLE I. Effects of Plasma from Anemic Patients upon Erythropoiesis in the Intact Rat.

Age, yr		Peripheral blood values of patient: Hb (g %)	Reties (%)	Nuc. RBC (per 100 WBC)		Effects in recipient rats (means ± S.E.): RBC (mill/mm³)	Hb (g %)	Hemat. (%)	Reties (%)	Marrow nuc. RBC (%)
I. Cooley's anemia										
5	♂	5.5	2.1	4	B*	8.82 ± .50	17.57 ± .87	46.6 ± 2.7	1.1 ± .30	
					A*	9.77 ± .74	18.03 ± .98	54.4 ± 2.0	6.7 ± .55	46.8 ± .2
10	♂	7.5	.6	3	B	8.90 ± .53	16.51 ± .15	47.5 ± .50	1.2 ± .35	
					A	10.30 ± .17	17.40 ± .21	49.0 ± 1.0	1.4 ± .40	45.5 ± 7.5
5	♀	5.0	1.1	0	B	8.05 ± .90	16.56 ± .90	47.7 ± .90	2.2 ± .30	
					A	10.24 ± .21	19.26 ± .43	59.0 ± 1.1	11.3 ± 1.40	51.5 ± 3.5
5	♀	7.0	2.5	4	B	8.11 ± .57	17.75 ± .28	49.6 ± .40	1.8 ± .50	
					A	9.17 ± .23	18.52 ± .35	52.5 ± 2.50	1.9 ± .40	32.5 ± 17.5
4	♀	6.0	3.5	13	B	8.41 ± .90	18.51 ± .35	52.8 ± 2.30	1.5 ± .60	
					A	9.85 ± .25	18.94 ± .08	60.5 ± 2.53	5.2 ± .70	44.5 ± 2.5
12	♂	8.0	7.2	240	B	8.79 ± .24	18.45 ± .56	51.1 ± .10	2.1 ± .50	
					A	9.06 ± .68	18.93 ± .76	57.2 ± 1.80	3.0 ± 1.10	45.5 ± 2.5
32	♀	9.5	6.0	1	B	8.42 ± .30	17.56 ± .65	49.6 ± 1.90	2.5 ± .19	
					A	9.51 ± .22	18.80 ± .31	52.5 ± 1.10	4.7 ± .64	43.6 ± 1.2
II. Sickle cell anemia										
11	♂	8.0	6.4	0	B				1.8 ± .26	
					A				5.6 ± .56	52.3 ± 1.2
15	♂	7.5	1.0	2	B	8.94 ± .28	16.36 ± .15	49.0 ± 2.01	1.9 ± .38	
					A	9.13 ± .34	17.38 ± .47	51.6 ± 2.06	2.4 ± .06	28.0 ± 2.7
III. Hypoplastic anemia										
7	♂	5.0	0	0	B	9.34 ± .22	18.22 ± .80	51.5 ± 1.34	2.2 ± .15	
					A	9.54 ± .47	18.86 ± .24	52.3 ± .93	3.8 ± .59	34.3 ± 4.1
IV. Controls										
12	♂	12.5	1.0	0	B	8.44 ± .70	17.15 ± 1.52	47.5 ± 1.11	2.6 ± .12	
					A	8.36 ± .36	15.93 ± .14	43.5 ± .95	3.6 ± 1.23	32.0 ± 1.0
12	♂	13.0	.4	0	B	8.61 ± .29	16.76 ± .24	50.6 ± 1.36	1.7 ± .62	
					A	8.77 ± .54	16.78 ± .02	49.8 ± 1.24	1.2 ± .03	27.5 ± 3.5
5	♂	12.0	.2	0	B	9.04 ± .09	18.27 ± .39	49.6 ± 2.17	2.0 ± .85	
					A	8.40 ± .28	17.26 ± .90	49.1 ± 1.19	1.8 ± .13	27.0 ± 3.0
6	♂	10.0	.4	0	B	8.75 ± .16	18.20 ± .15	48.8 ± .36	1.9 ± .21	
					A	8.65 ± .04	16.70 ± .34	46.6 ± .65	1.4 ± .15	33.5 ± 1.5
V. Cooley's anemia Urine extract										
(See above)		5.5	2.1	4	B				1.5 ± .23	
					A				3.6 ± .61	45.2 ± 1.1

* B—Before treatment; A—After 5 injections.

tralized with lN NaOH. In some cases saline was added during or after the boiling procedure in order to achieve a volume close to that of the original plasma. A similar acidification and boiling technique was used for the specimen of urine obtained from one of the patients with Cooley's anemia.

In view of the relatively small amounts of blood available, and to avoid the obviously inadequate procedure of pooling the plasmas of the different patients, no more than 2 to 3 recipient rats (young adult females of a modified Long-Evans strain) could be used in assessing EP activity in the plasma extract of each subject. Each rat received subcutaneous injections, daily for 5 days, of 1.8 to 3.0 ml of filtrate, equivalent in all cases to approximately 3.0 ml of original plasma. Five rats were employed to test the EP properties of the extract prepared from the urine of one of the Cooley's patients. Each rat received daily subcutaneous injections for 5 days of 1.5 ml of the urine extract, equivalent to 3.0 ml of the original urine. All rats were killed on the 6th day for erythrocytic studies of the peripheral blood and bone marrow as described

previously(7,22).

Results. It will be observed from Table I that in all cases, increases in the RBC count, hemoglobin and hematocrit values occurred in the rats receiving the plasma filtrates of patients with Cooley's anemia. Marked reticulocytosis was evident in Cases 1, 3 and 5, and strong stimulation of marrow erythropoiesis was noted with all extracts except that obtained from Case 4. With the latter extract, one marrow possessed 50% but the other only 15% nucleated RBC. Previous studies(11) have shown that marrows from 40 untreated rats of the same strain and sex possess 35.1 $\pm$ 1.9% nucleated RBC. A similar degree of EP stimulating activity, as judged by the reticulocyte and marrow response (only parameters examined), was revealed in the extract of plasma from one of the 2 cases (Case 1) of sickle cell anemia. The plasma extract from the second patient proved ineffective. None of the extracts obtained from the control donor subjects stimulated erythropoiesis in the recipient rats.

The extract of urine obtained from a child with Cooley's anemia (Case 1) also stimulated erythropoiesis, as noted from the significant reticulocytosis and the increased percentages of nucleated RBC in the marrows of the recipient rats (Table I). Unfortunately, in this case, the other peripheral erythrocytic parameters of the test rats were not ascertained.

The case of chronic hypoplastic anemia examined was a boy possessing this condition since birth. His RBC count, at the time of obtaining the peripheral blood sample was 1.8 mill/mm^3 and sternal biopsy indicated only 2% nucleated RBC within his bone marrow. Despite the intensity of anemia in this subject, no EP activity in the plasma extract was demonstrable in recipient rats (Table I).

Discussion. It seems clear that considerable EP stimulating activity exists in the blood of patients with Cooley's anemia. Activity was also present in the plasma of one of 2 subjects with sickle cell anemia. The EP factor detected here is heat stable and can be recovered in boiled filtrates of the plasma and urine. In these respects, it resembles the material present in the plasma of rabbits and rats subjected to hemolytic or hemorrhagic anemia(5,6,10,21). The activity of human circulating "erythropoietin" as seen in some of the patients (*e.g.* Cooley case 3), is actually greater than that detectable in any of the bled or phenylhydrazine-treated animals examined by us up to date, considering that the material of human origin was administered for only 5 days. It would thus seem that either human "erythropoietin" is somewhat different in chemical structure from the animal type or, what is more likely, it is the same substance but exists in larger concentration in the blood of the anemic patients studied. It was of interest that the extract of urine from one of the patients with Cooley's anemia possessed EP properties. Considering that the urine was concentrated to no more than 50% of its original volume, the reticulocyte and marrow response in the recipient rats represent a considerable degree of EP activity. Others(21) have detected EP activity in extracts of urine from rabbits subjected to bleedings. It is also apparent from the data that no correlation can be drawn between severity of the anemia in the patient at the time of blood withdrawal and intensity of the EP response to the plasma extracts in the recipient rats. Nor can it be related to the duration of the condition in the patient. Evidently the quantity of "erythropoietin" circulating in the blood is a variable one, dependent undoubtedly not only on the rate of production of the factor but also upon its rate of utilization or destruction. Any of these processes could conceivably change from time to time.

The results indicate that erythromicrocytosis, which is sometimes characteristic of the response to the plasma EP factor(10,11) was not apparent in the rats receiving the plasma extracts from the Cooley cases 1, 3, 5 and 6 (*i.e.* the percentage increase in the hematocrit values matched the percentage rise in RBC numbers). In most instances, hemoglobin production was not stimulated to the same extent as RBC proliferation, thus resulting in a lowered corpuscular hemoglobin content.

Since the blood of the subjects with Cooley's anemia displays strong EP stimulating

qualities and their marrows reveal marked erythroid hyperplasia, it would seem unlikely that a contributory factor to this condition is a reduction in the production or utilization of the EP factor. Undoubtedly, the more basic etiology centers about the abnormal hemoglobin and hemolytic mechanisms characteristic of these disease states(23-25). A significant finding was the absence of EP activity in the plasma extract of the patient with severe chronic hypoplastic anemia. It is possible that there is an impaired capacity of such subjects to elaborate the EP principle. Should this prove to be the case in more detailed studies of hypoplastic anemia, the administration to these subjects of purified "erythropoietin," preferably that obtained from human sources, would constitute a justifiable therapeutic measure.

Summary. Boiled filtrates of acidified plasma obtained from 7 patients with Cooley's anemia and one of 2 patients with sickle cell anemia stimulated erythropoiesis in intact rats. A similarly prepared extract of urine from one of the Cooley's subjects was also active. Plasma filtrates from normal subjects and one patient with chronic hypoplastic anemia were inactive. The implications of these findings are discussed.

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Effect of Route of Administration on Protective Action of Corticosterone and Cortisol Against Endotoxin. (22739)

HOWARD LEVITIN, MARY I. KENDRICK, AND EDWARD H. KASS*

Thorndike Memorial Laboratory, 2nd and 4th (Harvard) Medical Services and Diabetes Clinic, Boston City Hospital, and Department of Medicine, Harvard Medical School, Boston

The protective effect of cortisone (11 dehydro, 17-OH corticosterone) on lethal action of bacterial endotoxin(1-5) is in contrast to the usual adverse effects of cortisone and cortisol (17-OH corticosterone) on resistance to infection(6). This protective effect has been demonstrated in mice, rats and rabbits using endotoxin from several bacterial sources and its demonstration has generally required that the cortisone be given before or simultaneously with endotoxin(1-5).

Cortisone and cortisol have similar adverse effects on resistance to infection and on antibody production, but corticosterone, which is the predominant corticosteroid secreted by rats and, under ordinary circumstances, by rabbits, is less active than its congeners in these respects(7,8). It was therefore of interest to compare the actions of corticosterone and cortisol with respect to their effects on the lethal action of endotoxin.

Materials and methods. Animals. White male rats of a stock strain derived from the Sprague-Dawley strain, and weighing 130-180 g, were used throughout. They were fed a stock laboratory diet and water *ad libitum*. *Endotoxin.* The endotoxin was prepared from 15 liter amounts of culture of *S. typhi* (Ty 2), grown according to the method of Formal and Baker(9).† After overnight incubation 30 ml of chloroform and 250 mg of crystalline trypsin were added and allowed to act for 24 hours. Two volumes of acetone were then added and the sediment collected by centrifugation. The sediment was suspended in one liter of distilled water, the large particles removed by centrifugation and the crude endotoxin was precipitated out by addition to 2 liters of 95% ethyl alcohol and 1 liter of ethyl ether. The sediment was collected by centrifugation, resuspended in one liter of distilled water and again centrifuged. The supernatant was added to 1500 ml of ethyl alcohol and 750 ml of ether. The sediment was collected and suspended in 750 ml ether. The sediment was again collected, suspended in 750 ml of saline, centrifuged and the supernatant was stored at —20°C in small aliquots. Saline dilutions of this crude endotoxin were made prior to each experiment. Preliminary titrations (Table I) showed that 1.2 ml of this material per kg of body weight, injected intracardially in a volume of 1 ml, killed approximately 80% of rats; this amount was used unless otherwise indicated. The syndrome that followed the injection of endotoxin was the characteristic one that has been described(10). Mortality was recorded after 24 hours. In most of the experiments, the control animals received saline and alcohol in a volume equal to that of injected corticosteroid. However, when it became clear that the subcutaneous and intraperitoneal administration of saline and alcohol did not influence the reaction of the rats to LD_{80} of endotoxin, the use of control injections of saline was discontinued. In all experiments involving intracardiac injection, however, the control animals received saline and alcohol by the same route. *Corticosteroids.* Cortisol and

TABLE I. Lethal Effect of Intracardiac Injection of Endotoxin in Rats.

Amt inj., ml/kg*	No. surviving/No. inj.	% mortality
.8	3/ 8	62
1.2	5/21	76
1.6	0/ 8	100

* Endotoxin diluted in saline so that inj. dose was contained in a final volume of one ml. Saline alone given intracardially in 1.0 and 2.0 ml volumes to a total of 18 rats was not apparently deleterious.

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† We are indebted to Dr. E. E. Baker for his generous aid in the preparation of the endotoxin.

corticosterone were used as the free alcohols.‡ The crystals were suspended in 20% ethyl alcohol to the desired concentration and given in a volume of 1 ml by the subcutaneous or intraperitoneal route. Solutions for intracardiac use were made by dissolving the requisite amount of steroid in 95% ethyl alcohol and diluting with saline to a final concentration of 2.5%. Blood levels of cortisol were determined by a modification of the Porter-Silber method(11). Corticosterone concentrations were estimated by the method of Rosenman *et al.*(12). Although the methods are not completely specific the rates of disappeaarnce of the chromogens after the administration of the known steroid was taken to be a valid reflection of declining blood levels of the steroid. The data are presented uncorrected for endogenous chromogen. Blood for determinations of corticosteroid was obtained by exsanguination via cardiac puncture with the animals under light ether anesthesia. Blood from 2-4 rats was pooled in order to obtain sufficient serum for analysis and each point that is recorded represents a single pool. Corticotropin§ was given intramuscularly in oil and beeswax, as previously described(8). Lipoadrenal extract‖ and desoxycorticosterone acetate were given in oil intramuscularly.

Results. 1. *Effect of route of administration on protective effect of cortisol and corticosterone* (Table II). Corticosterone or cortisol were given subcutaneously in 10 mg doses and the animals were challenged at various times thereafter by the intracardiac administration of LD_{80} doses of endotoxin. Cortisol was protective when given 24 hours in advance of the endotoxin whereas corticosterone was not. However, when the time interval between the administration of hormone and of endotoxin was shortened, corticosterone became increasingly protective. When the hormones were given at the same time as the endotoxin, corticosterone was almost as active as was cortisol.

‡ Kindly supplied by Sharp & Dohme Division, Merck & Co.

§ Kindly supplied by Armour Laboratories.

‖ Kindly supplied by Upjohn Co.

TABLE II. Route of Administration and Protection by Cortisol and Corticosterone against Lethal Action of Endotoxin.

Route* and dose of hormone	Interval between hormone and endotoxin, hr	Cortisol	Corticosterone	Control
s.c., 10 mg	24	5/ 8†	0/ 8	1/ 8
	8	6/ 8	3/ 8	0/ 8
	2	8/ 8	6/ 8	3/ 8
	1	6/ 8	5/ 8	1/ 8
i.p., 10 mg	8	2/ 8	2/ 8	1/ 8
	.5	8/ 8	6/ 8	1/ 8
	−1	3/ 8	2/ 8	1/ 8
i.c., .5 mg	2	3/ 8	1/ 8	3/ 8
	1	5/16	2/16	5/16
	0	12/16	12/16	3/16

* s.c. = subcut.; i.p. = intraper.; i.c. = intracardiac.

† No. surviving/total No. inj.

After intraperitoneal administration, cortisol and corticosterone were not protective when the hormones were given 8 hours before endotoxin, but both were protective when given one-half hour before endotoxin. Intraperitoneal administration of steroid one hour after endotoxin led to markedly decreased protection as has been previously observed (5).

When cortisol or corticosterone were given simultaneously with endotoxin by the intracardiac route both steroids were active in protecting against endotoxin. The steroids were equally ineffective when given one hour or more before endotoxin by this route, indicating that the added steroid had probably disappeared from the blood in one hour.

II. *Rate of disappearance of steroid from blood of rats.* The differences in effectiveness of corticosterone and cortisol when given subcutaneously were evident when a relatively long interval existed between times of administration of steroid and endotoxin. To determine whether these differences were due in part to differences in rate of disappearance of the steroids from the blood stream, animals were given purified steroid by the intracardiac route. The rates of disappearance of corticosterone and of cortisol after 0.5 mg of each was given intracardially to rats were essentially the same (Fig. 1), if correction for the endogenous chromogen is made, suggesting

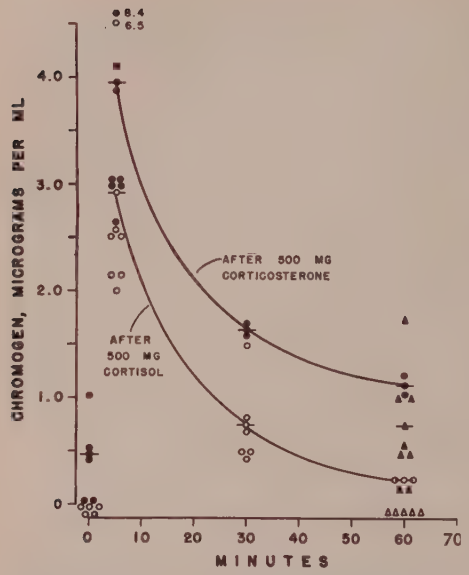


FIG. 1. Disappearance of cortisol and corticosterone from serum of rats after intracardiac administration. Solid symbols (●, ▲) refer to levels of 21-OH, 20 keto (corticosterone-like) chromogens, and open symbols (○, △) to 17,21 Di-OH, 20 keto (cortisol-like) chromogens. Triangles indicate blood levels of chromogens in control animals one hr after brief ether anesthesia had been given; circles show blood levels in resting control animals and following intracardiac inj. of either steroid.

that the differences in the effects of the 2 corticosteroids after subcutaneous administration were due largely to differences in their rates of mobilization from the local site of deposition.

III. *Protective effect of corticotropin and adrenal steroids against lethal action of endotoxin.* Corticotropin and lipoadrenal extract were protective against the lethal action of endotoxin (Table III), whereas desoxycorticosterone acetate was ineffective. Large doses of cortisol and corticosterone were, as previously mentioned, protective.

Discussion. The data demonstrate that corticosterone and cortisol were of similar effectiveness in protecting against the lethal action of endotoxin when the steroids were given directly into the blood stream at the time of administration of endotoxin. When, however, these steroids were given subcutaneously the protective effect of cortisol persisted for a longer period of time than did that of corticosterone. Since the rates of disappearance of these 2 steroids from the blood of the rats, after intracardiac administration, were similar, explanation of the differences in effects of these steroids must lie in the more rapid mobilization of corticosterone than of cortisol from the local site of deposition. Although differences in tissue mobilization or in rate of disappearance from the blood among steroids are well recognized(13,14), few experiments in which relative biologic activities of steroids are compared have taken these into account. It has been customary in experiments in this laboratory as well as in many others to regard corticosterone as having less biologic activity than cortisol or cortisone on the basis of the effect of widely spaced injections of hormone. It is apparent that many of the results of assays of the different biologic activities of these and related substances may require revision in the light of the findings herein reported.

That corticosterone and cortisol should prove to have similar activities when both are given directly into the blood stream is consistent with their close similarity in chemical structure and their occurrence singly or together as the predominant adrenocortical secretory products(15). Further study is required to determine if the reported differences between corticosterone and cortisol on inflammation and on resistance to infection and antibody formation(7,8) are related more to differences in mobilization of the steroid hormones from tissue sites of deposition than to major differences in their biologic activity when the steroids are presented to tissues at a constant rate.

The protective action of the corticosteroids

TABLE III. Protective Effect of Corticotropin and Adrenal Steroids on Lethal Action of Endotoxin.

Substance inj.	Dose*	No. surviving/No. inj.
Corticotropin	10 I.U.	7/8
DOCA	5 mg	2/8
Lipoadrenal	1 ml	5/8
Cortisol	10 mg	8/8
Corticosterone	10 "	6/8
Controls		3/8

* Test substance given intramusc. 48 hr, 24 hr, and 2 hr before inj. of endotoxin.

studied in these experiments is apparently related to increased concentrations of steroid in the blood during the time of exposure to endotoxin. Thus, one hour after intracardiac administration of corticosterone or cortisol, blood levels of corticosteroid had returned to normal values and the protective effect of the steroids was also absent.

The significance of the protective action of corticosteroid against endotoxin in terms of treatment is difficult to analyze. The protective effect was not demonstrable in dogs(16) or in guinea pigs(2,17); in animals in which it has been demonstrable, the effect was lost rapidly when steroid was given after endotoxin. In addition, Thomas and his coworkers(18) have shown that the prior administration of cortisone to certain rabbits prepared these animals for the development of generalized Shwartzman reactions after a single dose of endotoxin; the cortisone eliminated the need for the usual 2 doses of endotoxin. Only further study can determine whether corticosteroids influence endotoxic activity in established infections and to what extent corticosteroids alter the relationship between the adverse effect of corticosteroids on mechanisms of resistance to infection on the one hand, and the protective action of these steroids against endotoxin on the other.

Summary. 1. Both cortisol and corticosterone were effective in protecting rats against endotoxin when the steroid hormones were given intracardially at the time of administration of endotoxin. 2. Cortisol was more protective than corticosterone when both were given subcutaneously 24 hours before endotoxin; as the interval between the time of injection of steroid and the time of challenge with endotoxin was decreased, corticosterone was increasingly protective. 3. The rates of disappearance of corticosterone and cortisol from the blood of rats after intracardiac administration of steroid were similar. 4. The evidence indicates that corticosterone is more rapidly mobilized from local tissue sites of deposition than is cortisol and that the difference in the activity of these 2 substances after subcutaneous administration is due in large part to this difference. By inference, the many published differences in the biologic activities of cortisol and corticosterone may be subject to revision when control of this variable has been achieved. 5. Corticotropin and lipoadrenal extract were protective against endotoxin but desoxycorticosterone was not.

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Studies on Thymus of the Mammal. IX. Histochemical Study of Irradiated Mouse Thymus.*† (22740)

CHRISTIANNA SMITH
Department of Zoology, Mount Holyoke College, South Hadley, Mass.

The increased amount of lipids in the thymuses of C57 mice after exposure to a single total-body dose of x-rays was described by Smith and Holst(1). Thymuses normally contain little or no lipid. However, they become heavily loaded with fat 24 and 36 hours after irradiation when the evidences of injury are greatest. They return to a normal condition in 7-9 days. The mediastinal lymph nodes do not undergo a similar severe reaction. In the present investigation attention was directed to the study of acid phosphatase. In normal thymuses there is little indication of activity of this enzyme but after irradiation the changes in the number and distribution of cells showing sites of acid phosphatase activity correlate in general with lipid deposition.

Material and methods. BAlb/c male mice, 30-40 days old from the Roscoe B. Jackson Memorial Laboratory were used. The thymus, spleen and mesenteric lymph node were removed from control animals and from mice 2, 4, 8, 12, 24, 36 hours and 3, 5, 7 and 9 days after exposure to irradiation. The mice killed at 12 (in part), 24, and 36 hours had received doses of x-ray of 410 r; the radiation factors were 250 K.V., no filter, 3 min., 50 cm TSD.‡ The animals sacrificed 2, 4, 8, 12 (in part) hours and 5, 7 and 9 days received 434-455 r, gamma rays from radioactive cobalt (42 cm aluminum filter).§ Gomori's method(2) was used for the demonstration of sites of activity of acid phosphatase. The thymus, spleen and mesenteric lymph node were removed from each animal, fixed in ice-cold acetone and embedded in paraffin together; sections were cut at 8 μ and mounted on the same slide. The paraffin was not removed from the sections(3) and the incubation time in the substrate was usually 12 hours because this lengthened time brought out more clearly the slight reaction in the normal thymus. For cytological studies the period of incubation was reduced to 3 and 6 hours. The technic was controlled by incubating the sections in the buffer without the substrate. The two lobes of the thymus were often treated differently for comparative studies; (1) fixed in formal-calcium(4), embedded in 25% gelatin, sectioned on the freezing microtome and colored in oil red O; (2) fixed in acetone, embedded in paraffin and stained in periodic acid-leucofuchsin and methylene blue. Estimations of the amount of lipids were made independently by 2 or 3 persons by assigning values of + to ++++.

Observations. In studying the response of the thymus to injury the reactions of the mesenchymal and epithelial derivatives have been particularly noted. The nucleus of the former is characterized by a pattern of coarse chromatin; the latter by a large oval, vesicular nucleus, thin nuclear membrane and a fairly large bleb-like nucleolus to which is attached one or more small karyosomes. Thin cytoplasmic processes of the epithelial cells are well seen in Fig. 7 and 8.

The cortex of the normal thymus of the young BAlb/c mouse is less reactive than the medulla in all the technics used. In general there is no difference in the cortex between a subcapsular and an inner zone. There are few or no macrophages containing ingested cells, lipids or positive periodic acid-leucofuchsin granules. Only a few linear adventitial (mesenchymal reticular) cells along the capillaries contain lipids or give a positive re-

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action for acid phosphatase (Fig. 1). In the normal thymic medulla it is estimated that there are about 30% more lipids than in the cortex. Similarly there are more cells showing sites of acid phosphatase activity (Fig. 1). Macrophages with phagocytosed cells are scarce and small. These contain lipids and are blackened in the technic for acid phosphatase.

As soon as 2 hours after irradiation, even though the general appearance of the thymus was normal, the cells were visibly affected. In the cortex there was a limited amount of pycnosis. Phagocytosing macrophages were loaded with lipids and showed a positive reaction for acid phosphatase (Fig. 2).

At 4 hours after exposure the inner two-thirds of the cortex was more heavily laden with lipids than the subcapsular zone or medulla. In this region there were more lymphocytes and macrophages containing fat than at 2 hours and there was more evidence of acid phosphatase activity. Fig. 7 and 8 illustrate the characteristic appearance of the mesenchymal and epithelial reticular cells in the Gomori technic.

Degenerative changes were clearly evident 8 and 12 hours after irradiation. Localized cortical areas of pycnotic or fragmented nuclei gave little or no indication of acid phosphatase activity. In the inner cortical zone there were large macrophages loaded with ingested cells; these contained much lipid and gave a positive reaction for acid phosphatase (Fig. 3). In contrast to the cortex, the medulla showed little pycnosis, relatively less fat and fewer cells stained in sections treated for acid phosphatase.

At 24 hours post irradiation, the depleted and diminished cortex was heavily laden with lipids (about twice as much as at 12 hours) and stained a dark brown in the technic for for the demonstration of acid phosphatase (Fig. 4). The many loaded macrophages and epithelial cells were both colored in this reaction. Although there was a somewhat increased number of medullary cells which contained lipids and showed a positive reaction for acid phosphatase, the medulla was clearly less affected than the cortex.

Both cortex and medulla were estimated to be one-third to one-half more heavily laden with lipids 36 hours after exposure than at 24 hours and the cortex still stained deeply brown in the acid phosphatase technic. However, the removal of injured lymphocytes was indicated by the decreased amount of pycnosis and the greater number of vacuolated macrophages.

Thymuses examined 3, 5 and 7 days after irradiation showed that regenerative processes had started. At these times the amount of visible lipids and the evidence of acid phosphatase activity were diminished (Fig. 5). By 9 days the thymus was essentially normal (Fig. 6).

Discussion. Changes in lymphatic tissue after irradiation have been described mostly in relation to changes in the nuclei(5). More recently biochemical and histochemical technics have been applied to reveal other modifications in lymphatic organs after exposure to x-rays; for example, nucleic acids, Weymouth, Delfel, Doell, Steinbock and Kaplan(6); alkaline and acid phosphatases, Doyle(7,8); adenosine triphosphatase, Ashwell and Hickman(9); lipids, Smith and Holst(1).

The present study was concerned principally with the presence and distribution of histochemically demonstrable acid phosphatase in the thymus during its degenerative and regenerative phases after irradiation. The spleen and lymph node were similarly treated and examined for comparison (unpublished data). Acid phosphatase activity in the normal thymus is meager when compared with that evident in the red pulp and rim of the white pulp of the spleen or with the diffuse lymphatic tissue, medullary cords and sinuses of the mesenteric lymph node. This great difference probably results from the presence of many actively phagocytic cells in the spleen and lymph node and their scarcity in the normal thymus. As soon as 2 hours after irradiation there was an evident increase in acid phosphatase activity as the mesenchymal reticular cells became enlarged and rounded in both cortex and medulla (Fig. 2). They were, indeed, active macrophages with ingested lymphocytes. In these preparations

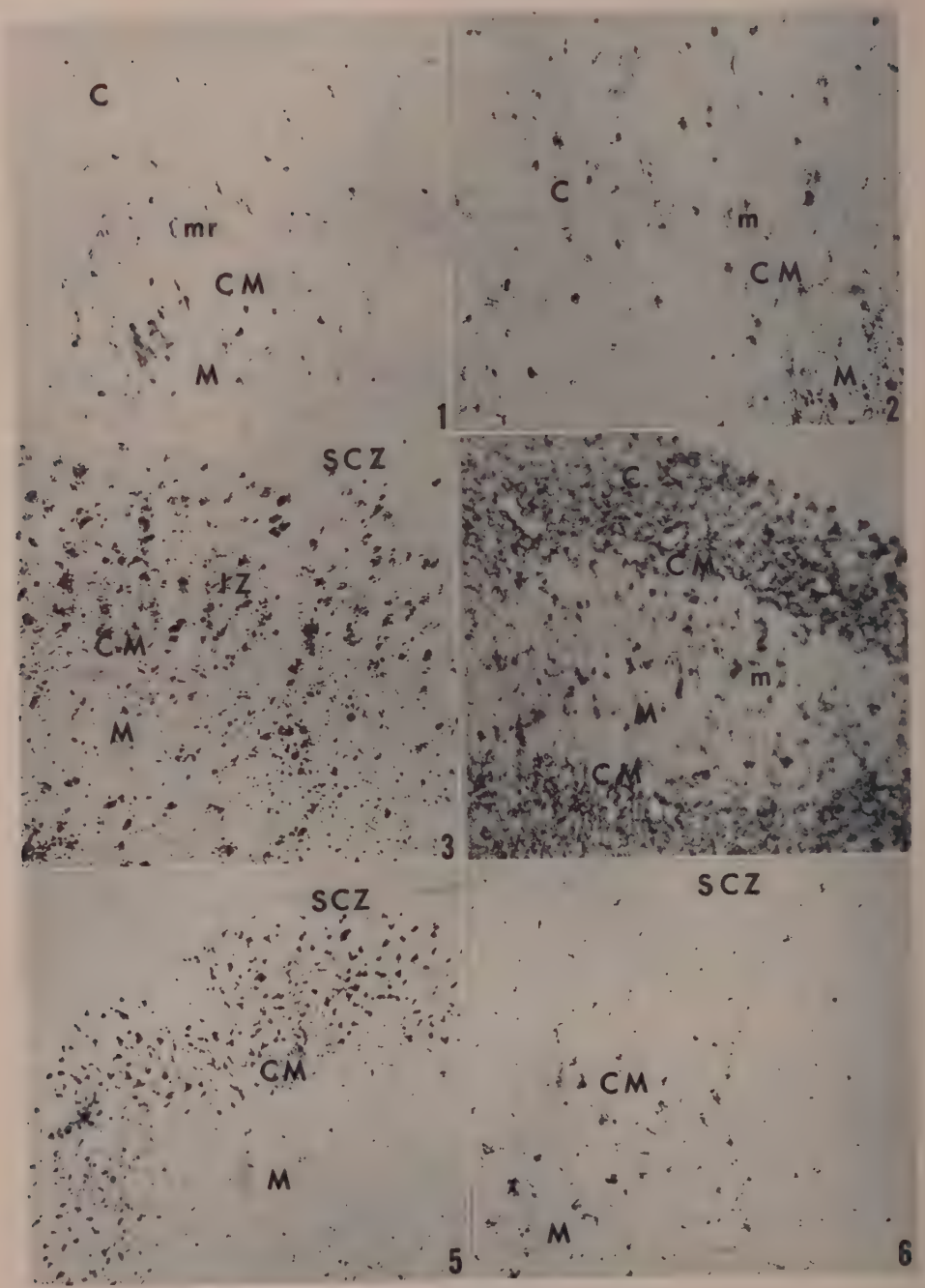


PLATE 1

Thymus of the mouse BAlb/c; fixed in acetone: Gomori's lead sulphide method for demonstration of sites of acid phosphatase activity; 12 hr incubation. × 100.

C, cortex; CM, cortical-medullary line; er, epithelial-reticular cell; IZ, inner zone; M, medulla; m, macrophage; mr, mesenchymal reticular cell; SCZ, subcapsular zone.

FIG. 1. Control. Linear mesenchymal reticular (adventitial) cells stained in cortex; slightly more stained cells in medulla.

FIG. 2. 2 hr after total-body irradiation. Development of macrophages seen as large round cells in cortex and medulla; few linear cells seen in cortex.

FIG. 3. 12 hr after irradiation. Large loaded macrophages more crowded in cortex than in medulla.

FIG. 4. 24 hr after irradiation. Narrowed cortex loaded with stained cells. Large macrophages seen clearly at edge of subcapsular zone. Vacuolated macrophages appear as empty spaces.

FIG. 5. 5 days after irradiation. Cortex, increased in width, contains more stained cells than medulla but macrophages are smaller.

FIG. 6. 9 days after irradiation. Thymus is essentially normal in appearance.

the lymphocytes which stained were near the blackened phagocytic cells. This quick mobilization of macrophages in the thymus is indicative of the rapidity with which thymic cells react to damaging influences. Kindred (10) noted that after the use of mustard gas the cortical macrophages of the thymus were active on the first day.

The indications of greatest activity of acid phosphatase were at the height of injury 24 and 36 hours after the sublethal dose of x-rays or gamma rays, the period also of the greatest deposition of lipids. The most vulnerable part of the thymus was the cortex; its inner zone was the first to show marked signs of injury. The intense staining at these hours (Fig. 4) was due to reactive macrophages, the epithelial cells and the coloring of the associated lymphocytes. Areas where there were dense accumulations of pycnotic or fragmented nuclei were ones where macrophages were scant or lacking. As compared with the cortex the medulla was much more stable during this period. In other technics this relatively quiescent medulla results in the appearance of the so-called inverted thymus.

Summary and conclusions. 1. In the normal thymus the relatively few mesenchymal

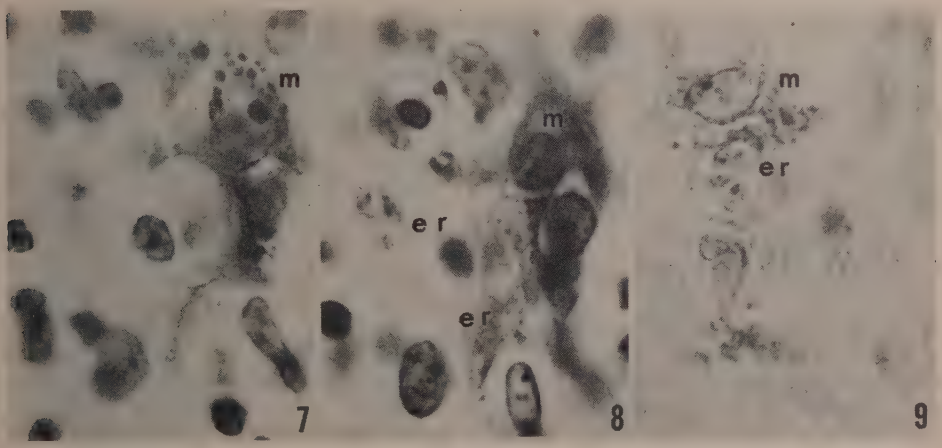


PLATE 2

Thymus of the mouse BAlb/c; fixed in acetone; Gomori's lead sulphide method for demonstration of sites of acid phosphatase activity; 6 hr incubation. × 1500.

FIG. 7 and 8. Medullary cells, 4 hr after irradiation. Same cells are photographed at 2 levels. Nuclei are stained with hematoxylin. Intimate association is seen of mesenchymal reticular cells with chromatic nuclei and stained inclusions in the cytoplasm and of epithelial cells with vesicular nuclei and weakly stained web-like cytoplasm.

FIG. 9. Medullary cells, 5 days after irradiation. A group of cells which include a macrophage with stained inclusions. Epithelial cells are barely visible. In this preparation only cells with stained cytoplasm had colored nuclei; lymphocytes and other cells with no evidence of acid phosphatase activity had unstained nuclei.

reticular cells give a positive reaction for acid phosphatase in acetone fixed sections. There are fewer reactive cells in the cortex than in the medulla. 2. As early as 2 hours after irradiation the mesenchymal reticular cells are mobilized to become active macrophages which stain deeply in the acid phosphatase technic. These become more numerous until at 24 and 36 hours after irradiation the involuted cortex is loaded with macrophages containing ingested lymphocytes or full of vacuoles. At these hours the browned cytoplasm of the epithelial cells is seen as the stroma of the depleted cortex. At 3, 5 and 7 days there is a gradual diminution in number and size of reactive cells. At 9 days the appearance of the thymus is essentially normal.

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Alterations in Calcium Metabolism in Cancer Patients Treated with 6-Diazo-5-Oxo-L-Norleucine.† (22741)

W. P. LAIRD MYERS AND GORDON B. MAGILL (Introduced by Joseph H. Burchenal)

Sloan-Kettering Institute, Memorial Center, and Department of Medicine, Cornell University Medical College, New York City

6-diazo-5-oxo-L-norleucine (DON), an antibiotic isolated from culture filtrates of a Streptomyces, has been reported by Clarke *et al.* (abstract) to inhibit the growth of sarcoma 180. It is also inhibitive in mouse leukemias and a spectrum of transplantable tumors. In combination with 6-mercaptopurine (6-MP) inhibition of transplantable tumors is synergistic. Patients with cancer metastatic to bone may demonstrate hypercalcemia and hypercalcuria secondary to the osteolysis incident to tumor growth. If the tumor growth is inhibited, as, for example, by local irradiation, the calcium changes may be reversed(1) with the cessation of bone breakdown. Hypercalcemia and hypercalcuria secondary to osteolytic cancer have been corrected also by the administration of adrenal steroids(2) and other compounds. These changes have been regarded as sensitive measures of decreased osteolysis secondary to a reduced rate of growth of tumor in bone(3).

In the course of clinical trials with DON, 11 patients with hypercalcemia and/or hypercalcuria secondary to osteolytic cancer were studied in order to detect any possible tumor inhibitory effects of the compound as measured by the sensitive changes in calcium metabolism. Marked effects on calcium metabolism were seen and it is the purpose of this paper to report these changes.

Material and methods. Six patients had carcinoma of the breast, 2 metastatic carcinoma of unknown origin, 2 bronchogenic carcinoma and one adrenal cortical carcinoma. Hypercalcemia occurred 13 times in these 11 patients. Treatment with DON alone was employed in 8 instances of hypercalcemia and the combination of DON and 6-MP was used in 5 instances. One patient who was normocalcemic but hypercalcuric was treated with DON alone. All but one patient were con-

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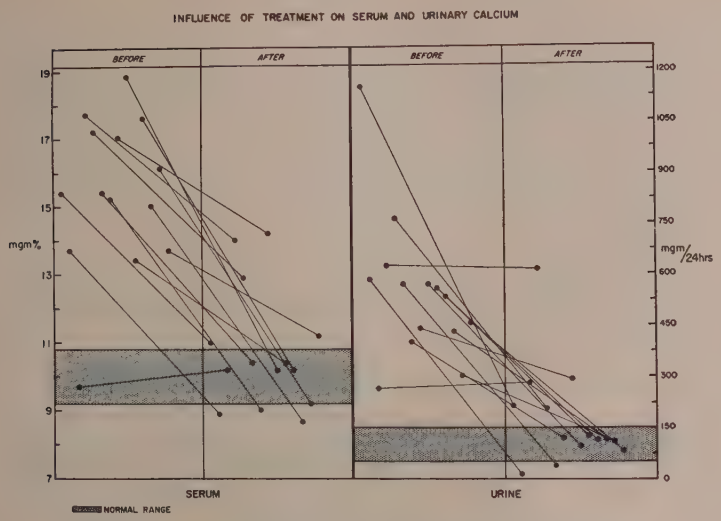


FIG. 1. Influence of treatment on serum and urinary calcium.

fined to bed and all had radiographically demonstrable metastatic cancer in their bones. DON was dissolved in saline in concentrations of 5 to 10 mg/ml. It was administered intravenously or intramuscularly in doses ranging from 0.1 mg/kg/day to 0.6 mg/kg/day. One patient received a dose of 1.2 mg/kg on one day only. Each daily dose was administered as a single injection. The duration of treatment ranged from 9 to 69 days. 6-MP was administered orally in doses of 2.5 mg/kg/day for 12-40 days. Treatment was continued in each patient until signs of toxicity developed (oral mucosal ulcerations, diarrhea, hematological depression).* Intravenous fluids had to be administered to most patients because they were too sick to retain oral feedings. Urinary calciums (method of Fiske and Logan)(4) were determined daily on 24-hour collections. Serum calciums (method of Clarke and Collip)(5) were analyzed approximately 3 times a week. The intake of calcium was restricted to 200 mg/day or less. A complete calcium balance was carried out in 2 patients. Stools and diets were ashed in a muffle furnace at approximately 600°C for 48 hours. Normal calcium ranges: Urine (on 200 mg intake) 50-150 mg/24 hours; serum 9.2-10.8 mg %. Full correction of hypercalcemia and hypercalcuria was regarded as having been achieved if the abnormal values returned to normal ranges regardless of the duration of such changes.

Results. (A) *General observations.* Fig. 1 illustrates the effect of treatment on the levels of serum and urinary calcium. The pre-treatment calcium levels represent the average of several determinations in all instances except when the serum and urinary calcium levels were rising sharply in the period before treatment. In these latter instances the highest pre-treatment value is illustrated. One patient was normocalcemic before treatment but all were hypercalcuric. All post-treatment values are the lowest ones achieved after initiation of treatment. They are not average values.

Serum. Reduction in the serum calcium occurred in each of the 13 instances of hypercalcemia although a return to normal or subnormal calcium levels occurred in only 8 instances (Fig. 1). No change from the normal range occurred in the single normocalcemic patient. The periods of treatment before these changes were seen varied from 7-26 days. The duration of the normocalcemic state was variable lasting from one day to as long as 26 days. In general, recurrences of hypercalcemia were observed when treatment

* Unpublished data.

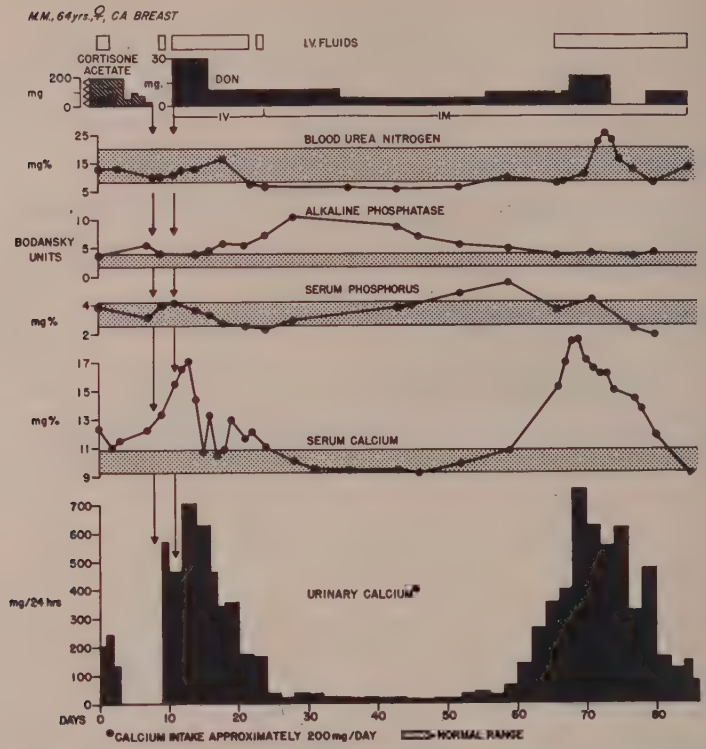


FIG. 2. Sequential changes in serum and urinary calcium in a patient with metastatic breast cancer under treatment with DON.

was stopped but the normocalcemic state persisted in some despite cessation of treatment and hypercalcemia recurred in one patient (Fig. 2) despite continuation of treatment. The decrease in the serum calcium often followed a "double-hump" pattern, that is, an initial decrease to near normal levels was followed by a rise despite continued treatment and then a fall to the normal range (Fig. 2). This pattern probably resulted from the practice of starting with high initial doses of DON (0.6 mg/kg/day) and then tapering to 0.2 mg/kg/day to avoid development of toxicity. In the 3 patients where the serum calcium decreased to lower than normal levels it rose again to the normal range although treatment was continued.

Urine. There was a decrease in the urinary calcium excretion in 12 of the 14 instances of hypercalcuria while 2 patients showed no change (Fig. 1). In 9 of these 12 instances the urinary calcium decreased to normal or lower than normal values. The pattern of response is illustrated in Fig. 2. In general the treatment periods required to produce these changes and the duration of the changes were similar to the serum calcium observations.

(B) *Treatment with DON alone.* There were three instances of full correction of both hypercalcemia and hypercalcuria. Two of these responses occurred in the same patient and are illustrated in Fig. 2. A near-complete response was observed in 2 additional patients whose serum calciums fell to 11.2 mg % and 11.0 mg % respectively with DON treatment. Definite hypercalcemia and hypercalcuria persisted in 3 patients but to a lesser degree than before treatment. There were no consistent differences in the dosages of DON received nor in the toxicity observed between those who responded and those who did not.

In general, the levels of serum and urinary calcium tended to rise promptly in both groups within 1-3 days after treatment with DON was discontinued. One patient, however, was an exception and remained normocalcemic for 22 days after cessation of DON therapy. One patient was normocalcemic but hypercalcuric (250-350 mg/24 hours) throughout the study and DON had no effect in lowering the already normal serum calcium. A total calcium balance was carried out in this patient and no effect on fecal calcium with DON treatment was noted. A total calcium balance was also carried out in another patient and no rise in fecal calcium was observed during DON therapy despite a decrease in the urinary calcium from 1148 mg/24 hours to 214 mg/24 hours over a period of 2 weeks. In one patient (Fig. 2) an attempt was made to prevent recurrence of hypercalcemia by continuing treatment with a maintenance dose of 0.1-0.2 mg/kg/day. Despite this, the hypercalcemia and hypercalcuria recurred but again were fully corrected by increasing the dose of DON.

(C) *Treatment with 6-MP plus DON.* Complete reversals of hypercalcemia and hypercalcuria were observed in 5 instances in the 4 patients in this group. The periods of treatment and total dosages of DON and 6-MP were approximately the same in each patient. In contrast to the DON group, the duration of induced normocalcemia (with one exception) ranged between 15 and 26 days and of normocalcuria between 6 and 32 days. One patient experienced only a 2 day remission in hypercalcemia but a second course of combination treatment resulted in normocalcemia lasting 19 days.

Discussion. The changes in serum and urinary calcium might be explained by a decrease in the rate of bone destruction by tumor owing to the inhibitory effects of the administered DON (and DON plus 6-MP). In view of the experimental evidences of tumor inhibition by these compounds and in view of other studies which suggest that correction of hypercalcemia is evidence of tumor inhibition in bone(1,2,3), this explanation seems most likely. The absence of roentgen evidence of bone healing is somewhat against such an interpretation and in one patient there was an actual increase in the extent of bone disease radiographically during the period between the two episodes of hypercalcemia (Fig. 2). Also disease which was present in soft tissue areas (liver, lung and subcutaneous areas) in some of the patients did not show any objective evidence of regression in conjunction with the calcium changes. All of the patients in this study succumbed to progressive disease and the transient clinical improvement noted in some was largely attributable to correction of hypercalcemia. In general, the addition of 6-MP to the treatment program seemed to exert a longer-lasting effect on the calcium changes noted. 6-MP alone has been used in 4 patients with hypercalcemia and/or hypercalcuria and no substantial effects on calcium metabolism have been observed. These observations are consistent with the demonstrated synergism between DON and 6-MP in transplantable tumors but further clinical studies are necessary in order to establish definite synergism.

Inasmuch as there was a decrease in urinary calcium excretion as the serum calcium fell rather than a calcium diuresis it is unlikely that the observed effects might have been due to enhanced renal clearance of calcium. One balance study revealed no rise in fecal calcium with DON treatment in spite of a marked decrease in urinary calcium, excluding, in this patient at least, the possibility of diversion of calcium excretion from urine to feces, as an explanation for the observed effects.

Chelation of calcium by DON was considered as a remote possibility but *in vitro* studies demonstrated no such action of the compound. Modes of action involving possible inhibition by DON of secretion of hypothetical bone-resorbing substances by the tumor remain entirely speculative at the present time.

Summary. Ten patients with hypercalcemia and hypercalcuria and 1 patient with normocalcemia and hypercalcuria were treated with 6-diazo-5-oxo-L-norleucine alone or in combination with 6-mercaptopurine.

Full correction of hypercalcemia with treatment occurred in 8 of 13 instances in these patients. Correction of hypercalcuria was observed in 9 of 14 instances. Various possible mechanisms underlying these observed effects are considered.

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Effect of Sodium Lack on Local Response of the Single Crustacean Motor Axon. (22742)

ERNEST B. WRIGHT* (Introduced by Hermann Rahn)

Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Sometime ago it was suggested, as the basic assumption for a theory on excitation of nerve (1), that a local or initial depolarization occurred in the immediate area of nerve to which the stimulus was applied. When this local voltage attained a certain optimum value it released or "triggered" the all or none response. Shortly thereafter this hypothetical local potential was measured directly(2) and examined in some detail(3) in crustacean nerve fibers. However, the actual source of this small potential remained obscure. Recently the all or none response has been studied quantitatively by these same investigators and others(4,5), and it has been established that the action potential is the result of an initial rapid sodium ion current inward through the fiber membrane followed by a slower outward potassium ion flow. The question now remains what is the cause of the small local disturbance always observed in the cathodal region with subthreshold stimulation? Is this also due to ionic currents or is it perhaps the result of the sudden appearance of a hormonal substance? It has been suggested, for example, that the release of ACH might produce the initial or local depolarization(6) as it does an end plate potential at the neuromuscular junction.

* Present address: University of Florida, College of Medicine, Gainesville.

The present work was undertaken to examine the local response of different single crustacean nerve fibers which possessed quite different excitability properties(7). A brief report of this investigation has already been given in abstract. Recently, certain results were obtained which, because of their significance, are described briefly in this paper.

Methods. The lobster single nerve fiber was used throughout. The preparation, mounting and recording technics have been described (8). The local or initial response was clearly observed with subthreshold stimulation when the fiber was entirely raised in oil, the V method, and stimulation and recording occurred in the identical cathodal area. Cole's physiological solution(9) was used with variations for the study of the effect of different ion concentrations.

Preliminary. One day by accident in mixing the physiological solution the buffer was omitted and although the pH was very low, found later to be only 5.8, the nerve fibers functioned well for several hours. Others, too, have noticed the lack of effect of low pH on these fibers (Adelman, personal communication). The importance of this finding however, was that it meant an entirely sodium-free solution could be applied to a nerve fiber, for some slight amount of sodium has always necessarily been included in the form of buf-

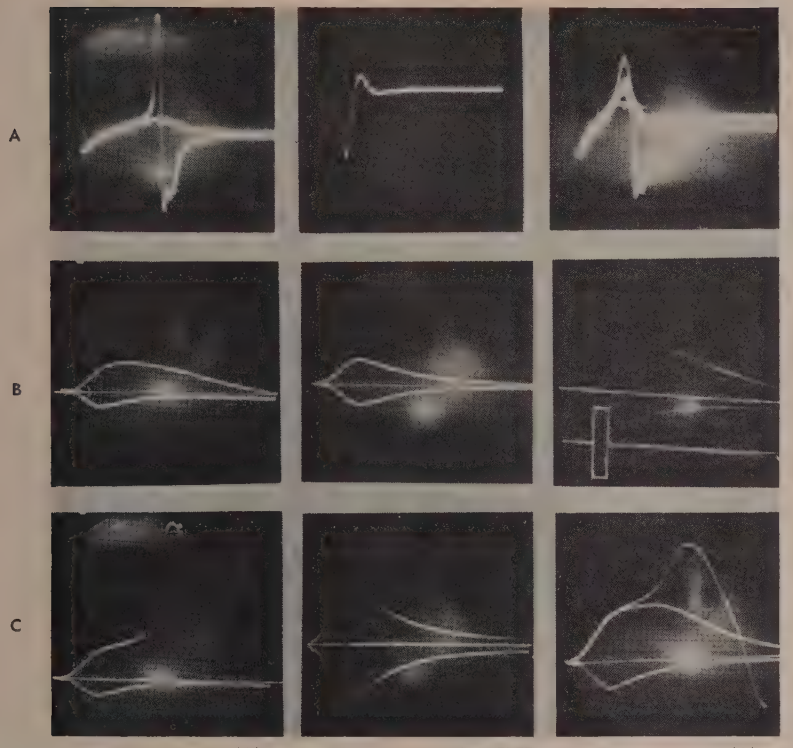


FIG. 1. Electrical response of a single crustacean opener fiber recorded directly at the stimulating cathode. Row A—Response with long duration, 100 ms, stimulation in normal physiological solution, left, sodium free (choline chloride substituted), middle, and normal solution, right. Row B—Recordings with short duration, 0.2 ms, stimuli of negative (upward) or positive (downward) polarity. Note asymmetry, negative deflection much larger in left and right pictures. In sodium free solution, middle picture, deflection is symmetrical. Row C—Same as B with stronger, threshold, stimulus. See text.

fers in so called "sodium free" media.

Results. When a single nerve fiber was dipped into a sodium free solution, containing dextrose or Choline Chloride to maintain the proper tonicity, all nerve electrical activity was extinguished instantly. Not only was the all or none spike immediately abolished, a not unexpected observation, but the subthreshold local response also disappeared at once. The effect was dramatically sudden and complete. With sodium ions added even in small concentrations the local response reappeared as abruptly as it disappeared in the sodium free medium. The recovery of the all or none potential was also extremely rapid but depended somewhat on the amount of sodium placed in the solution. When only a small concentration of sodium was added to the medium the spike appeared, not unlike the spurious response of a fatigued fiber.

A typical experiment is shown in Fig. 1. In the top row, A, the local and spike potentials elicited by "D.C." stimulation of 100 ms. duration are shown in normal, sodium free, and normal solutions, left to right respectively. The sweep speed for these recordings was slow. In the middle row, B, are illustrated the responses to just subthreshold, 0.2 ms. duration shocks. The sweep speed was fast. The bottom row, C, illustrates the recordings with threshold stimulation of 0.2 ms. duration in normal, sodium free and normal solutions, left to right respectively. The short duration shocks applied were both negative, upward deflection, and positive, downward deflection, polarity as shown in the

right picture, row B. A just subthreshold negative shock should produce a much larger deflection than a positive shock, due to the local response of the fiber itself in the neighborhood of a cathode(3).

At the start of the experiment with normal solution and D.C. stimulation there was a low voltage long duration subthreshold response and a large diphasic spike with threshold voltage. In the sodium free medium with strong stimulation there was a slight artifact but no real electrical activity. With sodium in the medium again, right hand picture, with varying strengths of stimulation, multiple sweeps, a large amplitude fairly short duration local response is the predominant deflection observed which when large enough produced an all or none response somewhat smaller and longer enduring than the original. Short duration subthreshold pulses normally elicited asymmetrical deflections, that is, the upward or negative deflection was far greater than the downward or positive deflection as expected (Row B, left picture). The negative deflection is augmented by the activity of the fiber, the local response, the positive deflection is not. The local response, catelectrotonus, of the fiber is responsible for the asymmetry of the deflections. These recordings are the same as described years ago(3). This asymmetry was also observed with threshold stimulation. However, with sodium free solution the responses due to positive and negative pulses, weak or strong, became perfectly symmetrical. The local response was gone, and only the shock artifacts of equal magnitude remained. The fiber was electrically totally inactive, or, to all intents and purposes "dead". With the return of sodium to the medium the action potential, slightly smaller and broader, appeared immediately and at the same time a subthreshold response of even greater asymmetry than at the start was recorded. This effect has been seen in all fibers used.

Conclusion. Sodium is necessary for the production of the local response. It has yet to be proven just what the role of the sodium ion is in the development of this local potential. The above findings do not eliminate the possibility of the release of a hormone such as ACH as the "trigger" mechanism, for it has been shown(10) that end plate potentials are all but abolished in the presence of ACH without sodium. However, it seems very likely that the sodium ion is the key to the setting off and development of the action potential. This has been known to be so for whole frog nerve preparations(11). Whether the ion actually moves inward to cause the local potential as it does during the rising phase of the spike remains to be determined. Its presence may simply be required for the initiation of some other reaction. The fact that the results are the same whether dextrose or Choline Chloride is substituted indicates that it is the sodium ion itself that is the essential particle.

Summary. The local or initial response of crustacean single motor axons has been recorded at the stimulating cathode. The deflections caused by a short duration shock of positive and negative polarity are asymmetrical. Normally the deflection caused by the negative pulse is considerably larger. In the absence of sodium, with either dextrose or Choline Chloride substituted, the local potential disappears. The deflections caused by positive and negative pulses become symmetrical. The effect is instantaneous and reversible. It is concluded that the sodium ion is essential for the production of the local or initial response.

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Propagation *in vitro* of Polioviruses. VIII. Effect of pH on Virus Yield and Cell Metabolism.* (22743)

GEORGE E. GIFFORD, HUGH E. ROBERTSON,† AND JEROME T. SYVERTON
Department of Bacteriology and Immunology, University of Minnesota, Minneapolis

Susceptible animal cell cultures infected by any of a variety of cytolytic viruses undergo progressive cellular degeneration(1). The pH of the culture medium is decreased concomitantly by cell metabolic activity. The purpose of this study was to measure quantitatively the effects upon virus yield and upon host cell metabolism of purposeful alterations in pH of the medium. Strain HeLa cells and poliovirus, Type 1, strain Mahoney, were employed to learn the effect of increments in pH from 6.3 to 8.0.

Materials and methods. Host cells: Strain HeLa cells were employed, cultivated, and maintained as described(2). The growth medium was composed of 40% human adult serum and 60% Hanks' balanced salt solution (HuS-40, BSS-60). *Assay for virus*: Virus titrations were conducted with 5 replicate HeLa cell cultures at each successive geometric dilution. Titers computed as most probable numbers of tissue culture infectious units (TCIU) from the frequency of positive tubes agreed within the range ± 0.3 log. For virus titrations the human serum growth medium was removed from cell cultures by 3 rinsings with BSS and replaced with 10% chicken serum, 90% modified maintenance solution (MMS) medium(3). *Virus*. Poliovirus, Type 1, strain Mahoney, was employed as supernatant fluid from passage 65 in cultures of HeLa cells, stored at —70°C. A small amount of virus (usually 15 to 30 TCIU) was employed in each experiment so that several cycles of virus synthesis could be followed and thus small effects would be magnified. *Manometric method*. The procedure for the cultivation of HeLa cells in Warburg flasks and the measurement of respiration has been described(4). *Glucose determination*. The Nelson-Somogyi photometric method of estimating glucose was employed(5). Readings were made in a Beckman DU spectrophotometer at 650 mμ. *Buffers*. For pH studies the MMS medium was fortified with additional buffers. Sodium hydrogen phosphite(4), 0.01 M, tris (hydroxymethyl) aminomethane (THAM), 0.01 M and sodium hydrogen phosphate, 0.01 M were employed. When buffers were employed an equivalent molar concentration of sodium chloride was omitted from the medium so as not to increase tonicity. The required pH was obtained by the addition of 0.15 M hydrochloric acid or sodium hydroxide.

Results. Effect of pH on poliovirus yield in stationary HeLa cultures. The first experiment was designed to test the effect of pH in range from 6.3 to 7.3 upon the replication of poliovirus in stationary cultures of strain HeLa cells. Supplementary buffers were found necessary to maintain the pH. Several buffers were tested for toxicity for strain HeLa cells. Table I shows that phosphate, 0.02 M, phosphite, 0.02 M and THAM, 0.01 M, did not significantly alter the respiration of HeLa cells over an observation period of 78 hours. Medium, ChS-10, MMS-90 containing 0.01 M each of phosphite, phosphate and THAM was prepared. Aliquots of the medium were adjusted to the required pH by the addition of 0.15 M HCl or NaOH, and were made up to equal volume

TABLE I. Effect of Various Buffers on Respiration of Strain HeLa in ChS-10, MMS-90.*

Buffer	Molarity	Oxygen uptake, μl, Hr of incubation 24	53	78
Control		212	475	751
Phosphate	.02	232	540	783
Phosphite	.02	235	550	800
THAM	.01	225	535	810

* 840,000 cells/flask.

* Aided by a grant from The National Foundation for Infantile Paralysis.

† Present address: Dr. Hugh E. Robertson, Cancer Clinic, Provincial Laboratories, Regina, Saskatchewan, Canada.

TABLE II. Effect of Variable pH upon Poliovirus Yield in Strain HeLa Cell Cultures.

pH			log TCIU/ml	
Initial	24 hr	37 hr	24 hr	37 hr
6.3	6.3	6.3	2.0	2.0*
6.5	6.5	6.5	2.5	3.5†
6.7	6.7	6.7	3.5	5.1‡
6.9	6.9	6.9	3.7	6.0
7.1	7.05	7.0	4.0	6.1
7.3	7.25	7.2	4.1	6.0

* Control bottles without virus demonstrated toxicity; over 50% of cells off glass, remaining cells rounded and granular.

† Control bottles without virus demonstrated toxicity; approximately 25% of cells off glass, remaining cells rounded and granular.

‡ Control bottles without virus demonstrated toxicity; few cells off glass, some rounding of cells.

with 0.15 M NaCl. Approximately 3.0 mg/ml of glucose were employed in the medium. Duplicate planar bottle cultures of strain HeLa were prepared at each pH increment and infected with approximately 25 TCIU of poliovirus. All cultures were incubated at 36°C. To better maintain the pH, 20% of the medium was replaced every 6 hours. The experiment was terminated at the first evidence of cytopathogenicity so that variations in the number of HeLa cells would not limit virus yields. Results of titrations of supernatant fluids harvested at 24 and 37 hours are shown in Table II. The virus yield from cells incubated at pH 6.7 or lower was significantly retarded or reduced. The possibility existed that decreased yield of virus observed at the lower pH levels might be due to a greater instability of the virus at these pH levels. Aliquots of media at pH 6.5, 6.8, 7.0 and 7.5 were prepared with $10^{3.8}$ TCIU/ml of virus and allowed to incubate at 36°C for a period of 42 hours. Titrations to determine the stability of poliovirus at these pH levels were performed. Titers all fell within the range $10^{3.5\pm0.3}$ TCIU/ml of virus. Maintenance of the original titer indicated that the virus was stable at the pH increments employed. *Effect of pH on HeLa cell metabolism.* To determine what effect alteration of pH had upon the metabolism of HeLa cells, quadruplicate Warburg flasks were prepared at each of 4 pH levels and 2 flasks of each series were infected with approximately 25 TCIU of virus. Medium, MMS-90, ChS-10, fortified with 0.01 M each of phosphate, phosphite and THAM, was employed. The results of three separate experiments are summarized in Table III. With alterations in pH from approximately 7.5 there resulted a decrease in oxygen uptake and a decrease in glucose utilization. The results at an initial pH of 6.9, 7.6 and 8.0 are shown in Fig. 1.

Discussion. Results of these experiments showed that the yield of poliovirus, Type 1, Mahoney strain, in HeLa cells was dependent upon the pH of the medium in which the host cells were maintained. The decreased yield of virus at decreased or increased pH levels was related to a decreased metabolic state of the cell cultures; such cultures failed to be maintained as shown by the decreasing rate of oxygen uptake. Fogh(6) investigated the effects of pH variation on absorption of poliovirus by monkey kidney cells; he concluded that possible differences in virus absorption at pH levels between 6.0 and 8.0 did not materially affect the latent period or virus yield. This evidence and evidence for the stability of poliovirus further supports the conclusion that reduced virus yield at low or high pH levels results from a decreased metabolic rate of the host cell. The present experiments also emphasize the importance of pH as a factor affecting metabolic rate of host cells and thus of virus yield. It is apparent that

TABLE III. Effect of Alterations in pH upon Poliovirus Yield and upon Metabolic Activity of Strain HeLa Cell Cultures.

pH		Glucose utilized,* μg/ml/10 hr/100,000 cells	Oxygen uptake,* μl/10 hr /100,000 cells†	Virus yield, log TCIU /ml
Initial	Final			
6.6	6.6	0	4.3	3.8
6.8	6.6	8	5.6	4.9
7.0	6.8	15	6.6	5.7
7.2	6.9	18	9.0	6.4
7.1	7.1	21	8.0	6.6
7.4	7.3	24	8.7	6.4
7.6	7.4	21	9.5	6.4
7.7	7.5	21	10.0	6.6
7.7	7.6	28	10.0	6.2
7.8	7.6	28	9.8	6.4
7.9	7.7	33	9.5	6.0
8.0	7.8	13	7.9	5.1

* Avg value obtained over period of observation.

† Initial live cell population.

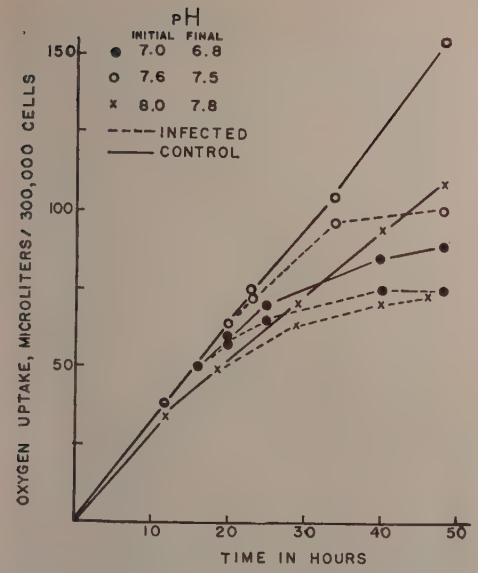


FIG. 1. Oxygen uptake of infected and control HeLa cell cultures at initial pH of 7.0, 7.6 and 8.0.

virus titrations should be carried out in adequately buffered media and that reduction of medium pH below 7.0 during incubation should be corrected. It is also apparent that in screening potential chemotherapeutic or chemoprophylactic agents the effect of pH should be considered. A test agent could act upon cells to increase glycolysis. The resultant increased acid production would thus lower virus yield to simulate specific interference with virus production.

Summary. The pH of tissue culture medium was altered purposefully to measure quantitatively the effects upon virus yield and host cell metabolism. By use of strain HeLa cells and poliovirus Type 1 (strain Mahoney) for growth in media of pH from 6.3 to 8.0 it was established that virus yield was affected by the pH of the culture medium. Decreased virus yield at decreased or increased pH levels was related to a decrease in metabolic rate of host cell cultures.

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Chronic Catheterization of the Renal Artery: Technic for Studying Direct Effects of Substances on Kidney Function.* (22744)

A. M. RUDOLPH,† S. N. ROKAW,‡ AND A. C. BARGER (Introduced by E. M. Landis)

Department of Physiology, Harvard Medical School, Boston, Mass.

During the course of studies on the pathogenesis of sodium retention in experimental congestive heart failure(1) it was shown that sodium retention occurred in dogs with normal basal glomerular filtration rates, suggesting the presence of increased tubular reabsorption of sodium. In order to obtain more direct information concerning the increased tubular reabsorption of sodium, a method has been developed for chronic catheterization of one renal artery, with collection of urine from each kidney individually. Such a technic affords the opportunity of studying the direct effect of salt solutions, hormones and drugs injected directly into the renal artery of the unanesthetized dog, using the opposite kidney as a control. Furthermore such experiments are relatively uncomplicated by extrarenal factors, and allow the separation of renal from

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† Fellow of the Amer. Heart Assn.

‡ Life Insurance Medical Research Fellow.

extrarenal effects in the unanesthetized dog. The presence of the catheter in the renal artery has not altered the glomerular filtration rate of that kidney, the renal plasma flow, the daily urine volumes, daily sodium excretions, or the response of the kidney to intravenous loads of sodium chloride.

Methods. Technic of urine collection from each kidney individually is a modification of the method described by Desautel.§ The dog, under sodium pentobarbital anesthesia, is placed in a supine position. Through a midline suprapubic incision the urinary bladder is exposed, and drained by needle puncture. The neck of the bladder is mobilized and the urethra cut between two heavy ligatures. The bladder is then hemisected in the sagittal plane, starting on the anterior surface, continuing up over the fundus and posteriorly down between the ureteric orifices. The hemisected bladder is then reconstructed into two separate bladder pouches. Each half of the bladder is sutured around a lucite funnel (Fig. 1A). Starting at the urethral end of the bladder the serosal surfaces of each half of the bladder are brought together by mattress sutures, inverting the bladder mucosa; a similar inverting suture is started at the fundal end of the bladder and continued down toward the midportion of the cut surface. The cup of the funnel is inserted into the bladder pouch thus formed and the sutures are continued up to the threaded stem of the funnel. A purse-string suture in the serosa is then drawn tightly around the funnel stem. The stem of each funnel is brought through a stab wound in the abdominal wall 2 to 3 cm lateral to the midline. Omentum is then sutured between the two pouches to reduce the likelihood of fistula formation, and the abdominal incision is closed. A lucite-nut with smoothed edges is screwed on to the stem of the funnel and tightened down to the abdominal wall to hold the bladder snugly against the anterior abdominal wall. A length of rubber tubing is attached to each funnel and is in turn connected to the plastic bags for urine collection (Fig. 1B). Rubber tubing is also attached to the distal end of

§ To be published.

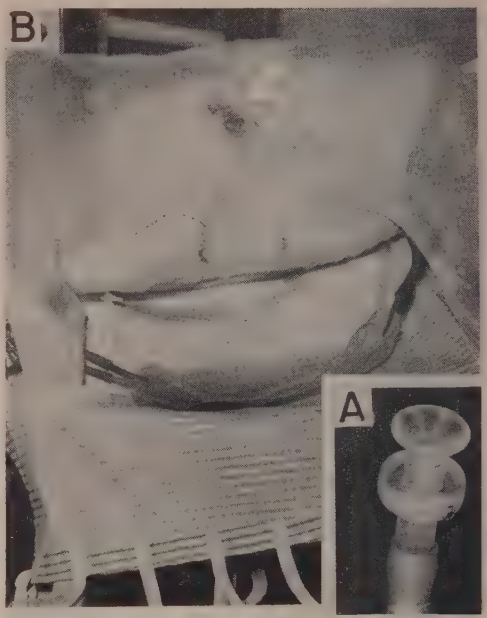


FIG. 1. A. Lucite funnel for urine collection. B. Illustration showing plastic bags attached to lucite funnels, and renal artery catheter secured to skin suture on flank of dog.

the urine bags and closed with Hoffman clamps, which allow fixation to the inside of the canvas jacket which is worn by the dog. The sterilized urine bags are changed daily. After a 7 to 10-day recovery period creatinine, para-amino-hippurate and sodium clearances are measured to assess any possible changes in these functions resulting from the subsequent catheterization of the renal artery. *Technic for implantation of catheter in renal artery.* The dog, anesthetized with sodium pentobarbital, is placed in the right lateral decubitus position. A left flank incision is made below the costal margin and the left kidney exposed. Blunt dissection in the retro-peritoneal space allows mobilization of the aorta for 2 inches above and below the left renal artery. The operation is abandoned if more than a single renal artery supplies the left kidney. Light rubber tubing is passed around the aorta, above and below the root of the renal artery, for counter traction and control of blood loss during subsequent manipulation. A 10-inch length of smooth, polyvinyl catheter is used. This catheter has an inside

diameter of .015 inch and an outside diameter of .028 inch with a total volume of approximately .05 ml. A stainless steel wire is threaded through the catheter to the tip to facilitate handling of the catheter. A purse-string suture is placed on the postero-lateral aspect of the aorta near the origin of the renal artery. The wall of the aorta is then perforated through this purse-string suture with a sharp needle. While countertraction is applied to the rubber tubes around the aorta, a blunt thin-walled curved needle, with a removal plug in the hub, is passed into the lumen of the aorta and across into the orifice of the renal artery and advanced approximately 1 cm. The plug is then withdrawn and the catheter passed through the needle and gently advanced beyond the end of the needle. The needle is cautiously withdrawn while the catheter is held firmly in place. The stainless steel wire is then removed. The purse-string suture around the catheter is then tied for hemostasis. The catheter is flushed with heparin and the external tip clamped. Branching of the renal artery usually occurs within the first inch of its origin from the aorta. To insure the perfusion of the entire kidney with injected substances, the catheter must be withdrawn until its tip lies proximal to the first bifurcation. Palpation of the catheter in the renal artery is not always reliable. Indigo carmine, injected through the catheter, produces a short-lived dark blue discoloration of the portion of the kidney through which the dye passes and sharply delineates the tissues not perfused with the dye. The catheter is then withdrawn 2 or 3 mm after each injection of indigo carmine until a single injection perfuses the entire kidney, indicating that the catheter tip now lies in the main stem of the renal artery and proximal to any bifurcation. The discoloration of the entire kidney also indicates that adequate mixing occurs in the renal artery. The catheter is then secured to the aortic wall. A small loop is left in the retroperitoneal space with further fixation to muscle and subcutaneous tissue. A second loop is left in the subcutaneous tissue and the external tip brought through the skin. The catheter is flushed with concentrated heparin (10,000 units per ml) and closed with a stainless steel obturator inserted into the tip. A fine tie is placed on the catheter over the metal obturator and this is secured to a loop sutured in the adjacent skin. A small protective covering is applied over the end of the catheter before the jacket is drawn on to the animal. The catheter is flushed each day with heparin.

TABLE I. Representative Daily Urine Volume (ml) of the 2 Kidneys before and after Chronic Catheterization.

Preoperative		Postoperative	
Left	Right	Left	Right
390	380	360	305
380	365	175	140
142	145	255	225
290	287	370	320

Results. Successful bladder division and catheter implantation has been performed in 5 normal dogs and 2 dogs with right-sided congestive failure. Such animals have now been followed for periods as long as 5 months without functional or pathological evidence of renal damage.

The ratios of the daily urine volumes of the 2 kidneys have not been altered by the insertion of the catheter into the left renal artery (Table I). Furthermore, the presence of the catheter has not modified the creatinine or par-aminohippurate clearances. In a representative dog (Table II) control creatinine clearances of the left kidney varied from 30 to 38 ml/min; after catheterization values ranged from 29 to 31 ml/min differences which are of the same order of magnitude observed in the right or control kidney. The para-aminohippurate clearances of the left kidney varied from 82 to 89 ml/min before,

TABLE II. Representative Creatinine and Para-aminohippurate Clearances (ml/min.) before and after Chronic Catheterization.

C_{CR}				C_{PAH}			
Pre-operative		Post-operative		Pre-operative		Post-operative	
Left	Right	Left	Right	Left	Right	Left	Right
36	32	29	28	82	79	83	85
38	37	30	31	89	87	90	94
31	28	31	33	85	80	94	95
30	28	31	32	85	81		

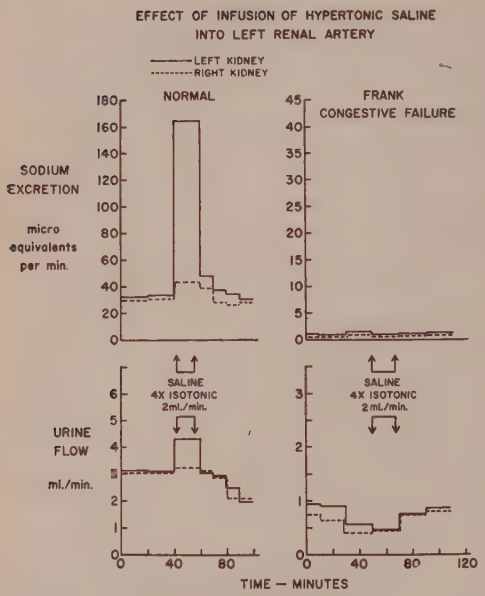


FIG. 2. Effect of infusion of hypertonic saline into left renal artery of normal dog, and dog with congestive heart failure.

and 83 to 94 after catheterization. Repeated clearance measurements over a period of 5 months have shown no significant changes. The animals were sacrificed at varying intervals after catheterization; neither the control nor catheterized kidneys showed any histological abnormalities. The bladder mucosa was hyperemic and hypertrophied.

With the successful development of the technic for chronic catheterization of the renal artery preliminary studies have been performed on renal tubular reabsorption of sodium in normal dogs and in dogs with chronic congestive heart failure. For example, as indicated in Fig. 2, the infusion of hypertonic saline (580 meq/L) into one renal artery of a normal dog at the rate of 2 ml/min produces a 5 to 10 fold increase in sodium excretion on the injected side with a smaller increment in water excretion. Little or no change is observed in the control kidney. A similar infusion into the renal artery of a dog in congestive failure, with marked sodium retention but a normal basal glomerular filtration rate, does not produce any increase in sodium or water excretion—further evidence for increased tubular reabsorption of sodium and water in congestive heart failure.

Summary. A method has been developed for chronic catheterization of one renal artery, with collection of urine from each kidney separately. The presence of the catheter has not altered renal function over a period of months. Preliminary studies with infusion of hypertonic saline into the renal artery of the dog in congestive heart failure indicate a marked increase in tubular reabsorption of sodium.

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Adenosine and Ash as Unidentified Chick Growth Factors in Fish Meal.* (22745)

S. J. RITCHEY, H. M. SCOTT, AND B. CONNOR JOHNSON
Department of Animal Science, University of Illinois, Urbana

Growth stimulation of many microorganisms by adenine and other purines has long been recognized(1). It has also frequently been postulated that in animal nutrition it may be possible that the rate of purine synthesis is inadequate for optimal growth, and at various times adenine and its derivatives have been stated to possess vitamin-like properties. For example, vit. B_4 has been postulated to be adenine and vit. L_2 has been stated to be adenylthio-methyl pentose. Huff and Bosshardt(2) have reported that the growth retardation brought about by the inclusion of succinylsulfathiazole in the diet of mice is

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largely prevented by the addition of adenine and by other purines and purine ribosides. Very recently(3) it has been reported that adenosine stimulates the growth of chicks fed a purified diet. A number of reports indicate that the ash of various natural products will, under certain conditions, stimulate the growth of chicks on purified diets(4-6) and corn-soya type rations(7). In contrast, negative results from alfalfa ash(8) and the ash of distillers' dried solubles(9) have been reported with purified diets where the salt mixture used in the present work was employed.

Fractionation work on the unidentified growth factor(s) in fish meal has been under our investigation for some time. We are presenting evidence at this time that the organic factor in fish meal is not adenosine and that supplemental adenosine does not improve our basal diet which gives excellent growth in contrast to the growth reported in the study referred to above. The fish meal† employed has consistently given a marked growth response when added to a purified basal diet containing an isolated soya protein.

Methods. Male chicks from a mating of New Hampshire males x Columbian females were used throughout. The parent stock had received a diet containing liberal amounts of fish meal, whey, and alfalfa meal. The chicks were housed in electrically heated batteries with raised wire floors. They were offered food and water *ad libitum* from one day of age. In each experiment three or four replicates of 10 chicks were used for each treatment. The experimental ration is given in Table I. The fish meal ash was prepared by ashing in a muffle furnace at a dull red heat and the ash was fed at a level equivalent to the ash in 10% fish meal. This amounted to 2% ash added to the ration. All supplements were added at the expense of cerelose. The general experimental design of the 3 experiments reported here can be seen from Table II. In Exp. 2 the Glista salt mixture was increased from the usual 5.34% to 7.34% in group 4, and in group 5 it was decreased to 3.34% so that the 2% fish meal ash replaced 2% of the usual mineral mixture. In Exp. 3 adenosine was fed both as the only supplement to the diet (group 4) and also on top of added fish meal (group 5) and on top of added fish meal ash (group 6).

Results. From the results presented in

TABLE I. Composition of Basal Diet.

	%
Cerelose	57.11
Drackett protein C-1	35.30
Salt mixture*	5.34
Corn oil (refined)	1.00
DL-methionine	.75
Glycine	.30
Choline Cl	.20
Vitamins†	+
Total	100

* See reference 8.

† Vitamins (mg/kg diet):

Thiamine HCl	100	Inositol	100
Niacin	100	P-aminobenzoic acid	2
Riboflavin	16		
Ca-pantothenate	20	Ascorbic acid	250
B_{12}	.02	Menadione	5
Pyridoxine HCl	6	α-toc. acetate	20
Biotin	.6	Vit. A acetate	10,000 I.U.
Folic acid	4	D_3	600 I.C.U.

† A defatted fish meal prepared by VioBin Corp, Monticello, Ill., and generously supplied by Mr. Ezra Levin.

TABLE II. Growth Response of Chicks to Various Supplements.

Group ration	Avg wt (g) 28 days	% increase over basal
Exp. 1		
1. Basal	280*	—
2. " + 10% fish meal	355	26.8
3. " + 2% fish meal ash ≅ 10% fish meal	307	9.6
Exp. 2		
1. Basal	381†	—
2. " + 10% fish meal	438	15.0
3. " + 2% " " ash	389	2.1
4. " + 2% Glista salts	390	2.4
5. " − 2% Glista salts + 2% fish meal ash	395	3.7
Exp. 3		
1. Basal	365*	—
2. " + 10% fish meal	425	16.4
3. " + 2% fish meal ash ≅ 10% fish meal	379	3.8
4. " + adenosine 150 mg/kg	346	−5.2
5. " " + fish meal (2 + 4)	414	13.4
6. " " + ash (3 + 4)	358	−1.9

* Avg of triplicate groups of 10 chicks each.
† " " quadruplicate " " " " " .

Table II it appears evident that adenosine gave no growth response when added to the soy protein type synthetic ration used in this study, or when added to this diet on top of fish meal or fish meal ash supplements. The picture with regard to ash is not quite so clear but certainly, considering all experiments, little response was obtained compared to the response obtained by the addition of 10% of this particular sample of fish meal. Other experiments not reported here have given the same order of response with respect to the intact fish meal.

Summary. It was found that adenosine at 150 mg/kg of ration failed to stimulate chick growth when supplementing a 30% protein, soy protein type purified diet. Supplementation of such rations with 2% fish meal ash gave only small and variable responses, while supplementation with 10% fish meal uniformly gave marked growth response.

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Effect of Replacement Therapy on Dental Caries Experience of Radiothyroidectomized Rats.* (22746)

JOSEPH C. MUHLER, DAVID BIXLER, AND WILLIAM G. SHAFER
Indiana University Department of Chemistry and School of Dentistry, Bloomington and Indianapolis, Ind.

Previous work from this laboratory(1-3) has indicated that the thyroid gland in rats is associated with the dental caries experience. By feeding desiccated thyroid it has been possible to repeatedly demonstrate a significant reduction in dental caries while the administration of I^{131} or thiouracil results in a significant increase in dental caries. From these data it appears quite convincing that the thyroid gland is intimately associated with the dental caries experience in rats. It has become a difficult problem to determine accurately the mechanism by which these effects take place. Various facts are known, however, which suggest that the salivary glands are important intermediates in this mechanism. For example, when salivariadenectomized rats are used, there is no appreciable anticariogenic effect observed by feeding desiccated thyroid. Also, it has been possible to demonstrate a direct relationship between thyroid function, the histological structure of the submaxillary gland and the incidence of dental caries. A further correlation has been shown not only between the microscopic structure of the submaxillary glands, their proteolytic enzyme content and dental caries incidence in hypophysectomized rats but also of proteolytic activity and arginase activity in the submaxillary glands with thyroid function(4-6).

To extend these studies on the relationship of the rat thyroid gland and dental caries, it was considered important to determine if it were possible to return the increased incidence of dental caries occurring in radiothyroid-

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TABLE I. Comparison of Incidence of Dental Caries in Radiothyroidectomized Rats Receiving Thyroxine and Testosterone.

Group	Sex	No. of animals	Final wt (g)	No. of lesions		Probability*	
Control	♂	21	370	8.9	> 8.8	—	.001
	♀	18	225	8.7			
I^{131}	♂	18	185	12.7	>13.5	.001	—
	♀	19	131	14.3			
Control + thyroxine	♂	16	326	6.8	> 6.1	.05	.001
	♀	17	242	5.4			
Control + thyroxine + testosterone	♂	14	332	5.4	> 5.9	.05	.002
	♀	15	261	6.4			
I^{131} + thyroxine	♂	18	340	7.4	> 7.5	.11	.01
	♀	19	229	7.6			
I^{131} + testosterone	♂	15	201	11.5	>11.6	.01	.05
	♀	17	178	11.7			
I^{131} + thyroxine + testosterone	♂	15	331	8.6	> 8.3	<.5	.02
	♀	16	252	8.0			

* Probability was calculated from No. of lesions with sexes combined, and the probability values should be compared only within each column.

ectomized rats to that of the control value by replacing the thyroid hormone. Thyroxine administered subcutaneously was used for this purpose instead of desiccated thyroid in the diet as used in previous studies since by use of injection technics it is possible to accurately regulate the dosage. Inasmuch as testosterone has been shown to be necessary for the histological maintenance of the submaxillary gland(6), it was also included in these studies.

Materials and methods. A total of 280 weanling Sprague-Dawley strain rats were divided into 7 experimental groups according to sex and initial body weight. Four groups received 500 μc of carrier-free I^{131} by subcutaneous injection; the first of these groups received no hormone supplement while the second, third and fourth groups received respectively 50 μg of thyroxine daily by subcutaneous injection, 2.5 mg/week/animal of testosterone propionate in croton oil injected intra-muscularly and a combination of thyroxine and testosterone at the same levels as used above. Two non-thyroidectomized groups received hormone injections, one receiving thyroxine and the other testosterone at the same levels as used in the radiothyroidectomized rats, while a final group received no radioiodine or hormone supplements and served as a control group. All of the animals were housed in raised screen cages and received the same cariogenic stock corn diet† (Fluorine = 0.5 μg/g) and fluorine-low (F = 0.5 μg/ml) drinking water. The duration of the experiment was 140 days after which time the animals were sacrificed by ether, the heads removed for dental caries evaluation by methods previously described(7) and the salivary glands and thyroid glands fixed for histologic study.

Results. The results of these experiments are seen in Table I. The injection of radioiodine results in a significant increase in the incidence of dental caries and confirms previous studies(3). Similarly, when thyroxine was injected into non-thyroidectomized rats the incidence of dental caries was significantly decreased. When both thyroxine and testosterone were injected into non-thyroidectomized rats the dental caries incidence was essentially the same as the non-thyroidectomized group which received thyroxine.

The injection of thyroxine into thyroidectomized rats produces a significant decrease in the caries rate and results in the same incidence of dental caries as found in the con-

† The composition of diet used was as follows: yellow corn grits, 52.7%; ground yellow whole corn, 11.3%; powdered whole milk, 30.0%; alfalfa, 4.8%; iodized sodium chloride, 1.0% and irradiated yeast, 0.2%.

trol group. Testosterone injection does not decrease the caries incidence to the same degree as thyroxine in thyroidectomized rats, but does result in significantly less dental caries than found in the non-hormone injected radiothyroidectomized animals. The injection of both thyroxine and testosterone into radiothyroidectomized rats results in approximately the same incidence in caries as found in the controls.

The thyroid glands of the animals which received radioactive iodine exhibited the expected microscopic findings of severe fibrosis with loss of recognizable follicles in most instances. The submaxillary salivary glands reacted to the thyroid disturbance in a manner similar to that following the administration of propylthiouracil. The granular tubules were markedly diminished in size and exhibited severe loss of granules in those animals which had been radiothyroidectomized. The administration of thyroxine alone or in combination with testosterone to radiothyroidectomized rats returned the structure of the submaxillary glands to that comparable to the control animals. Testosterone alone did not exhibit this ability. It may be concluded, therefore, that thyroxine itself or one of its metabolic products is responsible for maintenance of the submaxillary gland granular tubules, directly or indirectly.

Discussion. The relationship between the thyroid gland and the dental caries incidence is clearly shown in this study. When thyroxine is injected into non-thyroidectomized rats a significant decrease in the caries incidence results while radioiodide causes a significant increase in the incidence of dental caries. Thus, the amount of thyroxine used in this study and desiccated thyroid fed as a component of the diet used in previous studies appear to have about the same degree of anticariogenicity. The simultaneous administration of thyroxine and testosterone does not appear to have a synergistic effect on caries, and results in about the same decrease in dental caries as found by the injection of thyroxine alone.

The marked anticariogenic effect of thyroxine is best manifested in the radiothyroidectomized group. In this instance the administration of thyroxine results in a marked decrease in the dental caries experience. Testosterone also significantly decreases the caries experience in radiothyroidectomized rats, suggesting that both of these hormones are necessary for maintaining the normal salivary gland function in the rat. These studies seem to convincingly implicate the thyroid hormone in maintaining the function of the salivary glands and in turn directly affecting the incidence of dental caries in the rat.

Summary. The activity of the thyroid gland appears to be directly related to the incidence of dental caries in the rat, for thyroxine injected significantly decreases the caries rate and radiothyroidectomy significantly increases it. The thyroid hormone appears to be primarily involved since the increased caries rate resulting from radiothyroidectomy is directly counteracted by the administration of thyroxine. Testosterone similarly appears to play a role in this mechanism, since the dental caries rate is also significantly reduced when it is injected into radiothyroidectomized animals even in the absence of thyroxine.

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Effects of Penicillin G and Penicillin V on Thiamine Synthesis in the Rat.* (22747)

K. R. Johansson

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis

A few workers have related the growth promoting ability of antibiotics to elevated levels of certain limiting dietary vitamins in tissues and/or intestinal ingesta(1-6). In this study, an attempt has been made to correlate hepatic and intestinal levels of thiamine with the rate of growth of young rats fed rations containing a sub-optimal concentration of vit. B_1 and supplemented with one of three salts of penicillin G (benzyl penicillin) or with sodium penicillin V (phenoxymethyl penicillin).

Materials and methods. Forty weanling Sprague-Dawley albino female rats of between 40 and 50 g in weight were fed a semi-purified, thiamine-inadequate diet of the following percentage composition: "vitamin-free" casein (Nutritional Biochem. Corp.) 20, dextrinized corn starch 28, sucrose 39, hydrogenated vegetable oil (Crisco) 2.8, corn oil (Mazola) 4, salts (U.S.P. XIV) 4, L-cystine 0.2, and powdered cellulose (Alphacel, Nutritional Biochem. Corp.) 2. The following vitamins were added to the ration (mg/kg): thiamine hydrochloride 0.4, riboflavin 7.0, pyridoxine hydrochloride 5.0, calcium pantothenate 25, nicotinamide 25, i-inositol 100, choline chloride 1,000, folic acid 0.5, biotin 0.1, *para*-aminobenzoic acid 25, 2 methyl-1, 4-naphthoquinone 2.5, and vit. B_{12} 0.025. The corn oil additive contained 0.5 g oleum percomorphum and 125 mg d-alpha tocopherol/100 g. A portion of the carbohydrate component of this ration was in the form of dextrin because Sauberlich(7) reported antibiotics to overcome more readily a thiamine inadequacy if the dietary carbohydrate were dextrin than if it were sucrose. After a 5-day depletion period, groups of 4 animals were subjected to the following regimen:

I	Basal ration
II	*Idem* + potassium penicillin G
III	" + procaine penicillin G
IV	" + benzathine penicillin G
V	" + potassium penicillin V
VI	" + 5.6 mg thiamine hydrochloride/kg
VII	" + injections of potassium penicillin G
VIII	" + " " procaine penicillin G
IX	" + " " benzathine penicillin G
X	" + " " potassium penicillin V

Penicillin was incorporated in the diets at a level of 100 mg/kg ration or injected intraperitoneally (0.5 mg) once every 48 hours. Food and water were provided as needed and the animals were weighed at regular intervals. No record was kept of the amount of food consumed. On the 27th day of treatment, groups I-V were killed by a sharp blow on the head and their intestines and livers excised. The contents of each ileum and cecum were expelled into small screw-cap jars and frozen immediately in a dry ice chest. Each liver was weighed and also frozen. Each of the 3 types of specimens was pooled by ration group. On the 28th day the remaining animals (Groups VI-X) were killed and livers and intestinal contents recovered as described for Groups I-V. The thiamine content of each sample was determined subsequently by means of a microbiologic assay using *Lactobacillus fermenti* 36(8). The livers were thawed and dispersed in water with a Potter-Elvehjem homogenizer prior to assaying them for thiamine. The intestinal samples were thawed as needed, suitably diluted in water, mixed thoroughly and a portion centrifuged at 2,500 rpm in a Serval model SP/X centrifuge. The supernatant liquid was recovered and assayed directly while the portion of the suspension which had not been centrifuged was prepared for assay in the recommended manner by over-night digestion with pepsin and takadiastase. Thus, the intestinal contents were assayed for "free" and "total" thiamine. Just "total" thiamine was estimated from assays of the liver preparations.

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TABLE I. Effects of Oral and Parenteral Administration of 3 Different Salts of Penicillin G and One of Penicillin V on the Growth of Weanling Rats Fed a Thiamine-Inadequate Diet. Average 27-day net gain in body wt (g) ± stand. dev.

Treatment	Mode of administration of penicillin	
	Oral*	Parenteral†
K penicillin G (II & VII)	98.38 ± 7.57	88.25 ± 22.10
Procaine penicillin G (III & VIII)	91.13 ± 10.66	75.25 ± 15.99
Benzathine penicillin G (IV & IX)	99.00 ± 7.60	61.50 ± 14.72
K penicillin V (V & X)	88.25 ± 11.21	73.25 ± 11.62
Thiamine-insufficient control (I)	50.75 ± 21.20	
Thiamine-sufficient " (VI)	105.63 ± 7.14	

* By means of analysis of variance of these data (Group VI excluded from analysis) a highly significant difference exists. $LSD_{.01} = 26.4$.

† No statistically significant difference was found in these groups. Calculated as indicated in footnote *.

Results. The growth data assembled in Table I reveal that: a) the level of thiamine employed in the basal ration was not adequate for normal growth; b) supplementation of the deficient basal ration with excess thiamine resulted in normal growth rates; c) ingestion of penicillin stimulated growth significantly (indicative of an anticipated thiamine-sparing action); and d) intraperitoneal administration of the penicillins was not accompanied by a statistically-significant growth response. It is of interest that the three salts of penicillin G and the one of penicillin V were essentially equal in their ability to spare the dietary requirement for thiamine when given *per os*. It would appear that potassium penicillin G was superior to the other penicillins when administered parenterally, although the growth response was not significant when subjected to an analysis of variance.

The results of the thiamine assays of the livers are seen in Table II. Note the consistently higher concentrations of hepatic thiamine in rats given penicillin than in the deficient control rats (Group I). It is apparent also that the oral route of administration of penicillin was more effective than the parenteral one in enhancing storage of thiamine in the liver. When expressed as the amount of thiamine per liver, the stimulatory action of penicillin on the hepatic stores of vit. B_1 is even more striking than an expression of concentration. Hence, penicillin had an effect upon the size of the liver in these rats. Also, it would seem that potassium penicillin G was more effective in encouraging liver stores of thiamine than the other penicillins tested.

The data in Table III are not particularly illuminating in suggesting reasons for effects of penicillin on liver stores of thiamine. Note that in the ileum both "free" and "total" thia-

TABLE II. Effect of Penicillin on Weights and Thiamine Levels of Livers from Rats Fed a Thiamine-Inadequate Ration. Expressed as fresh wt of liver.

Treatment		Avg thiamine content		Avg liver wt (g)
		Conc. (μg/g)	Total (μg/liver)	
Thiamine-insufficient control		.84	4.27	5.11 ± 1.12*
Thiamine-sufficient "		2.82	21.09	7.48 ± 1.53
K penicillin G	Oral	1.54	10.76	7.01 ± .29
	Parenteral	1.64	9.93	6.04 ± 1.47
Procaine penicillin G	Oral	1.29	8.98	6.96 ± 1.21
	Parenteral	1.07	5.56	5.18 ± .96
Benzathine penicillin G	Oral	1.21	8.99	7.45 ± 1.11
	Parenteral	1.06	5.33	5.03 ± 1.17
K penicillin V	Oral	1.22	8.44	6.93 ± .91
	Parenteral	1.06	5.77	5.45 ± 1.08

* ± stand. dev.

TABLE III. Thiamine Levels in Ileal and Cecal Contents of Rats Given Penicillin and a Thiamine-Inadequate Diet. Results expressed as $\mu g/g$ wet wt.

Treatment		Ileum		Cecum	
		"Free"	"Total"	"Free"	"Total"
Thiamine-insufficient control		.34	.70	.38	1.00
Thiamine-sufficient "		.90	1.69	.63	2.68
K penicillin G	Oral	.17	1.57	.18	.76
	Parenteral	.005	.51	.31	1.37
Procaine penicillin G	Oral	.45	1.53	.03	.53
	Parenteral	.02	.52	.91	
Benzathine penicillin G	Oral	.19	.64	.30	.51
	Parenteral	.005	.47	.69	1.49
K penicillin V	Oral	.17	.60	.45	.85
	Parenteral	.005	.005	.83	1.47

mine existed at greater concentrations in animals given the antibiotic orally than in those which had received penicillin intraperitoneally. However, in no case was the concentration of "free" thiamine in ileal contents increased by the administration of penicillin; in only two cases (orally-administered potassium and procaine penicillin G) did penicillin raise the level of "total" thiamine in ileal ingesta. The only consistent pattern in cecal contents is that parenteral administration of penicillin, with but one exception, elevated the levels of "free" and "total" thiamine while oral administration of the drug had an opposite effect.

Discussion. This report adds weight to the accumulating evidence that antibiotics aid in the vitamin economy of certain animals. However, the manner by which this is achieved is unknown. True, the intestinal flora is affected(3,5,6), a fact which we felt unnecessary to establish in this report. Moreover, absorption from the intestinal lumen should be considered in view of reports that antibiotics cause a thinning of the intestinal wall(9,10). Unfortunately, our data on the influence of penicillin on the thiamine content of the intestinal ingesta are inconclusive. Nevertheless, the positive effect of penicillin on liver stores of thiamine may be a reflection of intestinal synthesis and absorption, a supposition advocated by others(2,4,9).

Apparently the different physical properties of the penicillin salts tested had little to do with the thiamine-sparing activity of penicillin, since the 3 salts of penicillin G and the one salt of penicillin V differed but slightly in their effects on the rate of growth. It is obvious further that incorporation of the antibiotic in the ration gave a growth response superior to that obtained with widely-spaced parenteral injections of penicillin. It must be remembered, however, that all of the penicillin given parenterally is excreted within 6 to 8 hours; thus the tissues of rats which received penicillin parenterally were exposed to penicillin approximately 8 of the 48 hours that elapsed between injections. On the other hand, bacitracin, an antibiotic that is poorly absorbed from the intestinal tract, has been reported to stimulate the growth of rats fed a vit. B_{12} deficient diet only when administered intraperitoneally(11). Presumably, if penicillin had been given to the rats parenterally every 8 hours instead of every 48 hours a growth response may have occurred. Despite the long interval between injections of penicillin, the total amount and concentration of thiamine in the livers of these animals was appreciably greater than in the thiamine-deficient control animals. Moreover, of the parenterally administered penicillins, potassium penicillin G enhanced the rate of growth as well as the liver's stores of thiamine to the greatest degree. Correlation of the growth rates with the content of thiamine in the livers and the liver weights is excellent (compare Tables I and II). Nevertheless, the thiamine-sufficient control rats had approximately twice the amount of thiamine in their livers than did the deficient rats showing the best response to penicillin.

Summary. Three salts of penicillin G and one of penicillin V were found to be essentially equal when administered orally in sparing the rat's dietary requirement for thiamine, as determined by rates of growth and thiamine levels in the liver. Growth was not stimulated to a statistically significant degree upon administering these antibiotics intraperitoneally every other day, although thiamine levels in the livers were elevated by such treatment. The effects of penicillin on thiamine levels in ileal and cecal contents were inconclusive.

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Effect of Stimulation of Adrenal Cortex on Blood Pepsinogen in Animals and Man. (22748)

ALVIN P. SHAPIRO, JULIAN MELHADO, ADACIE FOX, AND JAMES LAMM
(Introduced by Carleton Chapman)
Department of Internal Medicine, University of Texas, Southwestern Medical School, Dallas

The level of pepsinogen in blood and urine constitutes an index of gastric activity(1-4). Uropepsinogen excretion reportedly increases following administration of adrenocorticotrophic hormone or adrenal steroids, and after acute emotional and physical stress; therefore it has been suggested as a measurement of adrenal activity. These data and their implications concerning the relationship of gastric activity to stress and the General Adaptation Syndrome have been reviewed recently by Gray *et al.*(5). However, Hirschowitz and his associates were not able to demonstrate increases in blood pepsinogen following ACTH administration(6), and Necheles and his co-workers recently reported failure of cortisone to affect uropepsinogen excretion (7). Similarly, in animal studies, we were unable to demonstrate elevation in blood pepsinogen following either acute or chronic noxious stimulation in cats and rats even when adrenal hypertrophy was observed(8,9). A study was undertaken, therefore, to determine if direct stimulation of the adrenals by exogenous ACTH influenced blood pepsinogen in animals. The effect of a single injection of ACTH on blood pepsinogen in humans was also evaluated and titres in several patients on chronic steroid therapy were measured.

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TABLE I. Response of Blood Pepsinogen to Acute Adrenal Stimulation in Animals.

Animal	Treatment	Blood pepsinogen activity Before	After	Hr after inj.
Cats A	Saline	290	260	24
B	50 units ACTH gel	375	315	24
C	*Idem*	310	335	24
Dogs A	Saline	240	250	6
			260	24
B	40 units ACTH gel	490	350	6
			410	24
C	*Idem*	355	400	6
			405	24
Rats 1	Saline	260	275	24
2	"	250	205	24
3	10 units ACTH gel	265	320	24
4	*Idem*	—	210	24
5	Saline	260	245	48
6	"	285	375	48
7	"	300	255	48
8	30 units ACTH gel	—	325	48
9	*Idem*	320	240	48
10	"	345	340	48

Materials and methods. Acute studies were performed in 3 mongrel cats, 3 mongrel dogs, and 10 male Holtzman rats in whom blood pepsinogen was determined before and after administration of ACTH gel. Treatments and time of measurement of pepsinogen are given in Table I. Acute studies were also performed in 9 healthy human subjects, one patient with pernicious anemia and one with renal disease. In the latter condition, pepsinogen activity may be elevated, as has been demonstrated by Hirschowitz(3) and Spiro (10). In addition, pepsinogen was determined following chronic therapy in one patient with an inactive peptic ulcer and severe pulmonary disease, and in four patients without ulcers, who were receiving large doses of steroids and/or ACTH for a variety of ailments. Diagnoses, dosage schedules and experimental design in the human subjects are presented in Table II. To study adrenal cortical activity and blood pepsinogen levels simultaneously, 42 male Holtzman rats weighing 200-250 g were divided randomly into 4 groups. Blood was obtained by cardiac puncture, and the animals were treated for 5 days as outlined in Table III. All animals were then anesthetized with sodium pentobarbital and rapidly exsanguinated. Adrenals were cleaned of extraneous tissue, weighed to 0.1 mg on a Sartorius balance, and their ascorbic acid content determined by the method of Roe(11). Blood pepsinogen activity was determined by the method of Mirsky *et al.*(12). One unit of pepsinogen represents one microgram of tyrosine released by one ml of plasma or serum; plasma was used for the cats and dogs, serum in the rats and humans.

Results. A consistent increase in pepsinogen activity was not noted in the acute studies (Tables I and II). In 4 patients who were receiving chronic treatment with steroids, blood pepsinogen was within the normal range in our laboratory (*v.i.*) and rose slightly in only one of 3 with further treatment. Activity increased in the ulcer patient after one week of therapy with prednisone alone, but thereafter fell to initial levels despite continued administration of ACTH and prednisone (Table II). The effects of adrenal cortical stimulation on adrenal weight, ascorbic acid content and level of pepsinogen in rats are set forth in Table III. These data were subjected to an Analysis of Variance. The adrenals of the groups which received ACTH and epinephrine were significantly heavier and demonstrated a highly significant depletion of ascorbic acid, but pepsinogen activity was identical in all groups. Comparison of the pepsinogen values before and after treatment in each of the groups suggested a slight fall in the groups given ACTH and epinephrine, but this was not significant statistically. Carbaminoylcholine (Doryl), a parasympathomimetic agent with predominant gastro-intestinal effects, did not stimulate the adrenal nor elevate the average pepsinogen level; however 2 animals in the group displayed titres which were over 500 units. Peptic ulceration was not observed.

Serum pepsinogen levels determined in our laboratory in 18 normal patients ranged from 195 to 445 with a mean of 305 units, and a standard deviation of 78.5. In 24 patients with proven peptic ulcer, the values ranged from 366 to 968, with a mean of 610 and standard deviation of 162. Although there is

TABLE II. Response of Blood Pepsinogen to Adrenal Stimulation in Human Subjects.

Acute studies (11 subjects)

Sex	Treatment (ACTH gel IM), units	Blood pepsinogen activity Before	24 hr after	Sex	Treatment (ACTH gel IM), units	Blood pepsinogen activity Before	24 hr after
♀	40	300	340	♂	40	435	530
♂	40	295	300	♂	40	280	285
♂	40	290	240	♀	40	205	200
♂	40	205	290	♂*	40‡	50	60
♀	40‡	305	300	♀†	40‡	735	440
♂	40	250	210				

* Patient with pernicious anemia. † Patient with nephrotic syndrome and blood urea nitrogen of 50 mg %. ‡ Aqueous ACTH IM.

Chronic studies (5 patients)

Diagnosis	Treatment	Pepsinogen activity	Time (day of study)
Inactive peptic ulcer; chronic pulmonary disease	(1) Prednisone daily from day 0 to 60 (total dosage 490 mg)	890	0
		1150	8
		900	16
	(2) ACTH gel IM twice weekly from day 17 through 60 (total dose 260 units)	955	23
		905	30
		905	60
Drug sensitivity	(1) 5 to 10 mg Prednisone daily for 3 mo prior to day 0	400	0
	(2) 40 units ACTH gel IM daily from day 0 to 4	495	4
	(3) No therapy; day 4 to day 8	425	8
Undiagnosed collagen disease	15 mg Prednisone daily for 13 days prior to day 0 (total dosage 195 mg)	275	0
Rheumatoid arthritis, drug-induced Cushing's syndrome with marked hypertension	(1) Cortisone 25 mg daily for 20 days every mo for 3 yr prior to day 0	390	2
	(2) 40 units ACTH gel IM day 0 to day 7; 25 mg cortisone day 0 to day 2; 12.5 mg daily days 3 and 4	220	6
Drug sensitivity	(1) 40 units ACTH gel IM and 10 mg Prednisone; day 0	193	3
	(2) 40 units aqueous ACTH I.V.; day 1 and 2		

TABLE III. Comparison of Blood Pepsinogen Activity and Adrenal Cortical Activity in Rats.

Treatment*	No. of rats	Means and stand. dev. Adrenal wt (mg/100 g body wt)	Adrenal ascorbic acid (mg/100 g adrenal)	Pepsinogen activity (μg tyrosine released/cc serum) Before	After
Control	10	16.7 ± 2.1	455 ± 26.9	256 ± 79	251 ± 163
ACTH	11	22.6 ± 3.7	333 ± 53.9	264 ± 75	210 ± 99
Doryl	11	15.3 ± 1.7	462 ± 41.6	236 ± 85	264 ± 168
Epinephrine	10	19.8 ± 1.8	367 ± 38.9	257 ± 51	198 ± 75
p values Between groups		<.01	<.01	N.S.	N.S.†
Control × ACTH		.001	.001		
Control × doryl		N.S.	N.S.	Grand mean (before) 253 ± 74	
Control × epinephrine		.04	.001		

* Dosage schedules (all treatments of 5 days duration):

Control, 1 ml normal saline daily; ACTH, 10 units of ACTH gel daily; Doryl, 1.5 μg twice daily; Epinephrine, 0.4 mg (in oil) daily.

† Differences between grand mean before treatment and means of individual groups after treatment, including apparent declines in ACTH and epinephrine groups, similarly are not significant.

an overlap in the range from 350 to 450, the differences between means is highly significant ($p = .001$). In 5 patients with pernicious anemia the mean value was 137. The patients in these 3 groups all had normal levels of blood urea nitrogen. In 9 patients with blood urea nitrogen over 20 mg % (range 20 to 135), pepsinogen levels averaged 615 with standard deviation of 213. These data are reported to add our results to the literature confirming the significant elevation of blood pepsinogen in peptic ulcer, its depression in pernicious anemia, and its increase in renal failure.

Discussion. Failure to produce an increase in blood pepsinogen consistently following acute administration of ACTH indicates that the level of this enzyme cannot be considered a sensitive and reliable index of adrenal cortical activation. The studies in rats in which pepsinogen and adrenal cortical activity were measured simultaneously clearly indicate that stimulation of the adrenals sufficiently intense to cause hypertrophy and depletion of ascorbic acid, will not raise blood pepsinogen and may perhaps lower it. Nor was an appreciable elevation of blood pepsinogen noted in human subjects receiving chronic or large doses of ACTH and/or steroids. Accordingly, an elevated level of blood pepsinogen need not indicate adrenal hyperactivity, nor should it be interpreted as evidence that the General Adaptation Syndrome is causally linked to the development of peptic ulcer. These considerations do not obviate the fact that prolonged administration of ACTH or cortical steroids may produce an acute ulcer in some patients, after which pepsinogen may rise(6). However, in such instances the adrenal hormone probably plays a permissive, rather than a causal, role and operates by increasing the sensitivity of the gut to stimuli previously not damaging(13). Direct tissue changes, vascular disturbances, or changes in the character of the mucous secretion of the stomach represent possible mechanisms for this action, which need not then be associated with a prior rise in pepsin secretion.

The increase in *urinary* pepsinogen following ACTH or cortical steroids reported by Gray(5) is not explained by our data. Hirschowitz has demonstrated that gastric pepsin in the human does not increase regularly after ACTH while blood pepsinogen is essentially unchanged, and suggests that the increased urinary output reflects increased renal clearance and not increased production(6). These circumstances should of course result in an actual fall in blood pepsinogen, which is suggested by our data in rats and in the patient with impaired renal function whose blood level declined after ACTH. Accurate delineation of this problem however requires simultaneous measurement of urine, blood, and gastric pepsin under a variety of conditions. The precise meaning of changes in levels in blood and urine might still be obscured because the volume of blood considerably dilutes the small quantities of pepsinogen available for renal clearance, and the methods for measurement carry an inherent error of 5 to 10%. Accordingly, until these arguments are clarified, it would seem well advised to consider blood pepsinogen only as an indicator of gastric activity which is fairly constant in most individuals, elevated in patients with peptic ulcer, and not influenced by acutely stressful situations. Changes which occur in chronic situations with deep-seated psychodynamic dislocations as described by Mirsky(14), probably have no direct relationship to adrenocortical stimulation.

Summary. Blood pepsinogen levels were measured in cats, dogs, rats, and man following acute and chronic administration of adrenocorticotrophic hormone and/or cortical steroids. Despite demonstration of adrenal cortical stimulation, pepsinogen activity did not increase. The data indicate that blood pepsinogen activity does not constitute an index of adrenal cortical activation.

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Chromatographic Separation of Growth Hormone and Other Peptides.* (22749)

M. S. RABEN (Introduced by E. B. Astwood)

New England Center Hospital and Department of Medicine, Tufts University School of Medcine, Boston, Mass.

A chromatographic system was devised for the purification of growth hormone which appears, in addition, to be generally useful for the fractionation of polypeptides. The system may be adapted to either of two means of separation. The difference in maximal acetic acid concentration allowing adsorption on carboxymethylcellulose is sufficient to permit separation of some substances. Fractions which have similar adsorbability from acetic acid are separated by elution from carboxymethylcellulose with weak solutions of neutral salts in acetic acid. The resolving power of the salt elution appears to be particularly high when the acetic acid concentration is rather near the maximum strength permitting adsorption. The appropriate acetic acid concentration may be predetermined by small batch experiments, or by gradient elution from a column with the acid.

Results. Experiments with a purified growth hormone preparation indicated that adsorption occurred in acetic acid in strengths up to approximately 3.5 M. Gradient elution with acetic acid separated a minor component, but most of the material appeared with a sharp front at about 3.6 M but with extensive tailing and no further fractionation. Gradient elution with calcium chloride in 1.0 M acetic acid eliminated the tailing and yielded

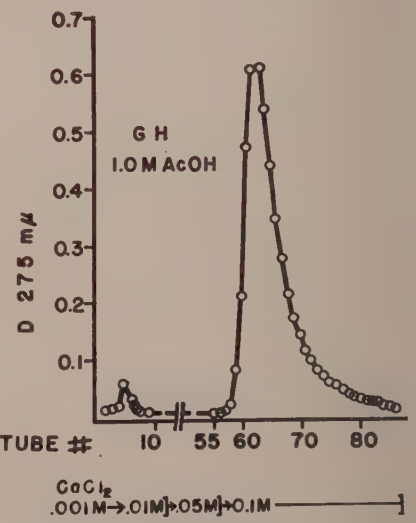


FIG. 1. 30 mg pig growth hormone preparation in 1.0 M acetic acid containing .001 M $CaCl_2$ on 3.5 g carboxymethylcellulose prewashed with same solution and packed under pressure. Column dimensions: 9 × 220 mm. Mixing flask for the gradient elution contained a constant vol of 120 cc. Flow rate, 7.4 ml/hr.

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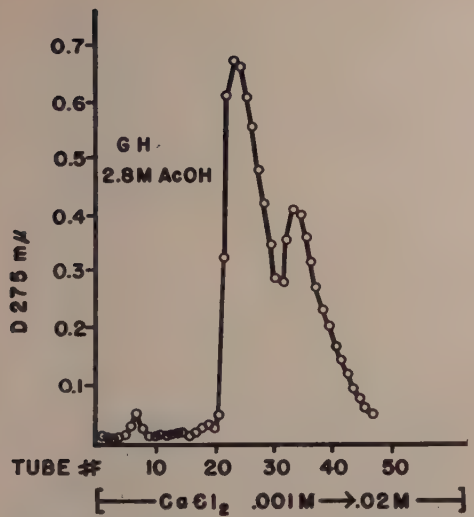


FIG. 2. Same as Fig. 1, except for changes in acetic acid and calcium chloride concentrations. Flow rate, 5.2 ml/hr.

one minor and one major fraction (Fig. 1); elution with calcium chloride in 2.0 M acetic acid separated the major fraction into two components, and the separation was somewhat better with 2.8 M acid (Fig. 2). The growth-promoting activity was concentrated in the faster running of the two components.

There was an inverse relation between acetic acid concentration and the concentration of salt required for elution. Elution with lanthanum chloride rather than calcium chloride decreased the required molar concentration of salt by about one-half. Gradient elution with calcium chloride in 0.3 M formic acid failed to separate the two major components, suggesting that the solvent character of the strong acetic acid solutions may contribute to the fractionating power of the system. However, the addition of .01 M calcium acetate to acetic acid prevented separation of the major components by gradient elution with calcium chloride, presumably due to the buffering effect. In general, resolution appeared to be improved by conditions favoring a weakened affinity of the adsorber for the peptide, and this may account for the impression obtained by Ellis and Simpson(1) that the resolving power of carboxymethylcellulose for growth hormone preparations was not particularly high under their conditions which were chosen to favor maximum adsorption.

Little, if any, growth hormone was bound irreversibly by the adsorbent, but loss of other protein material to the column may occur. The recovery from these columns was about 80%, but with another growth hormone preparation (Armour R285-128) slightly less than half the charge emerged under the same conditions. Application of a larger charge of the Armour material on a fresh column or reuse of the same column for the same charge yielded a major fraction of growth-inactive material appearing with the front and a growth-active fraction in the anticipated position.

Applying the information obtained from these studies, it was found possible to prepare growth hormone directly from a crude pituitary extract either by gradient elution, or by fractional elution with fixed concentrations of calcium chloride in 2.0 M acetic acid.

A purified corticotropin preparation was not adsorbed by carboxymethylcellulose from a 2.0 M solution acetic acid, but considerable fractionation was obtained by gradient elution with calcium chloride in a 1.0 M acetic acid solution (Fig. 3). Intermedin activity was concentrated in the third fraction and corticotropin activity in the unresolved fourth and fifth fractions. The small amount of spermine present in the preparation(2) was concentrated in the region of the second peak,

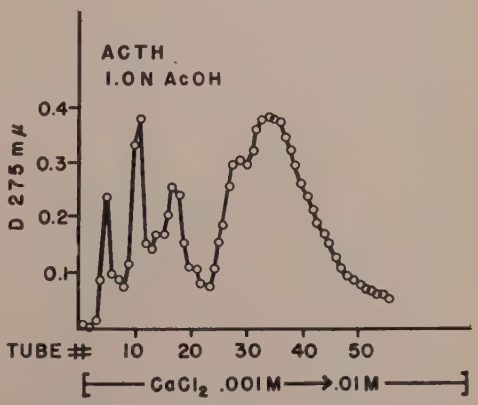


FIG. 3. 24 mg oxycel-purified corticotropin on column similar to those of Fig. 1 and 2. Flow rate, 6.4 ml/hr.

as judged by the formation of characteristic picrate crystals.

The carboxymethylcellulose used in these experiments was prepared from Solka-Floc BW according to the directions of Peterson and Sober(3) and contained approximately 0.75 milliequivalent carboxyl group per gram. The growth hormone was prepared from pig pituitaries as previously described(4), and the corticotropin preparation was purified by adsorption on oxidized cellulose as described by Astwood *et al.*(5).

It is of interest that carboxymethylcellulose adsorbed practically none of the corticotropin preparation from 2.0 M acetic acid but adsorbed 68 mg/g of the growth hormone preparation, since it was previously noted that 10.4% carboxyl oxidized cellulose adsorbed, from 0.1 M acetic acid, 340 mg/g of the corticotropin and only 6 mg/g of growth hormone(4).

Summary. A chromatographic system of high resolving power was devised for the purification of growth hormone and was adapted for use in the separation of other peptides.

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Effect of Allergic Shock on Fate of Staphylococci in the Organs of Mice. (22750)

SAMUEL J. PRIGAL AND RENÉ J. DUBOS

Department of Medicine, New York Medical College, Flower-Fifth Avenue Hospitals and Laboratories of Rockefeller Institute for Medical Research, New York City

As recently shown by several investigators (1,2,3) an anaphylactic type of sensitization can be elicited in mice by injecting intraperitoneally certain protein antigens in association with pertussis vaccine. The preliminary results here reported indicate that a mild allergic shock induced in animals so sensitized can modify their resistance to bacterial infections.

Experimental. Specific sensitization of mice to bovine serum. The ability of mice to develop an anaphylactic type of sensitization to bovine serum is illustrated in the following experiments with two different breeds of mice. Female mice of so-called Rockefeller Swiss strain were used when 7 weeks old; their average weight was 31 g. They were fed bread and milk throughout the experiment and sensitized as indicated in Table I. The bovine serum was a sterile filtrate distributed by Difco laboratories. The pertussis vaccine was a preparation distributed for human use by Lederle Laboratories and obtained through the kindness of Dr. Stanton M. Hardy. It contained approximately 60 billion bacterial cells/ml. The animals were challenged in groups of 5 by intravenous injection of various amounts of serum. All deaths occurred within 30-60 minutes (Table I).

Irrespective of the amount of serum used for the challenge injection, there were no deaths among the mice pretreated with either physiological saline, or with pertussis vaccine alone (Table I). In contrast, many of the animals that had been pretreated with serum died shortly after the challenge injection, even when the challenge dose was smaller than 0.01 ml. There was evidence furthermore that the degree of sensitization had been increased by injecting pertussis vaccine in addition to bovine serum at the time of sensitization.

The following experiment carried out with another strain of mice provides evidence that

TABLE I. Effect of Pertussis Vaccine on Sensitization of Mice* to Bovine Serum.

Sensitizing treatment (i.p.)		Challenge† (i.v.)	Deaths‡
Serum	Pertussis vaccine	ml serum	(out of 5)
ml			
.5	.05	.1	5
		.01	5
		.003	2
		.001	3
.5	0	.1	3
		.01	2
		.003	1
0	.05	.1	0
		.01	0
Saline controls		.1	0
		.01	0

* Female Rockefeller Swiss mice, 7 wk old.
† Three wk after sensitization.
‡ All deaths occurred within 30-60 min.

the allergic state elicited by serum-pertussis mixtures is specific. Female mice of the CF_1 breed were obtained from Carworth Farms and maintained on a diet of bread and milk. Individual weights ranged from 20-28 g when they received the sensitizing pretreatment. The bovine serum and pertussis vaccine were the same preparations that had been used in the preceding experiment. Fourteen days or 19 days after pretreatment, the animals were challenged with various amounts of bovine serum, rabbit serum, or egg albumin, in a final volume of 0.2 ml (Table II).

The results presented in Table II show that following treatment with bovine serum and pertussis vaccine, CF_1 mice developed a degree of sensitization comparable to that developed by mice of the Rockefeller Swiss breed. With both breeds of mice, intravenous injection of an amount of serum as small as 0.001 ml administered approximately 3 weeks after sensitizing pretreatment was sufficient to cause acute anaphylactic-like death (within 30-60 minutes) of a certain percentage of animals. The specificity of the reaction is indicated by the fact that there were no deaths among animals pretreated with pertussis vaccine alone and challenged with bovine serum, or among animals that had been sensitized with the mixture of bovine serum and pertussis vaccine, but were challenged with rabbit serum or egg albumin.

Effect of allergic shock on fate of staphylococci in the organs of mice. It has been observed in preliminary tests, not reported here, that the susceptibility to tuberculosis of mice sensitized to bovine or rabbit serum can be increased by injecting the tubercle bacilli in association with small amounts of the type of serum used for sensitization. As pertussis vaccine increases the ability of mice to develop the allergic state, an experiment was carried out to determine whether the concentration of this adjuvant used at the time of sensitization with bovine serum would influence the response of animals infected with bacteria resuspended in serum.

Four groups of female mice, 5 weeks old, of the so-called Rockefeller Institute Swiss breed, were pretreated by intraperitoneal injection of 0.5 ml bovine serum, with or without admixture of different amounts of pertussis vaccine (Sharp and Dohme). The preparation of pertussis vaccine used in this experiment was an experimental batch (not packaged for human use) which had been kindly supplied by Dr. J. Munoz of Merck, Sharp and Dohme. It must be noted that this preparation of vaccine was much more concentrated (approximately 15 times) than

TABLE II. Specific Sensitization of Mice* to Bovine Serum.

Sensitizing treatment (i.p.)		Challenge inj. (i.v.)		
Serum (ml)	Pertussis vaccine (ml)	Time after sensitization (days)	Material inj. (ml)	Deaths† (out of 5)
.5	.05	14	.2 bovine ser.	5
			.1	5
			.05	3
			.02	4
			.005	4
			.001	0
0			.2	0
0			.1	0
.5	.05	19	.01 *Idem*	5
			.001	3
			.001	1
			.0005	0
			.2 rabbit ser.	0
			.01 " "	0
			.2 1% egg alb.	0
			.05 " "	0

* Female CF_1 mice, 7 wk old.
† All deaths occurred within 30-60 min.

TABLE III. Effect of Amount of Pertussis Vaccine Used as Adjuvant on Susceptibility of Mice to Staphylococcal Infection.

Sensitizing treatment,* pertussis vaccine (ml i.p.)	Challenge infection† (i.v.): 0.1 ml staph. culture + 0.1 ml 1/1000 bovine serum										
	Deaths within 4 hr	No.‡ of staph. colonies recovered from individual organs									
		Kidneys					Lungs				
.02	3/5	2	12				4	10			
.006	0/5	1	70	400	1100	3000	10	10	10	300	300
.0006	1/5	5	100	1700	5000		100	240	250	3000	
0	0/5	11	17	100	300	1200	30	30	30	290	500

* All animals received 0.5 ml bovine serum i.p.
† 27 days after sensitization.
‡ Figures to be multiplied by 1,000 for whole lung, and by 10,000 for whole kidney.

the one from Lederle Laboratories used in the 2 preceding experiments. Twenty-seven days later, all animals received a mixture containing 0.1 ml of culture of *Staphylococcus aureus* (Smith strain) and 0.1 ml of 1/1000 bovine serum in saline. The characteristics and behavior of this staphylococcal strain, *in vitro* and *in vivo*, have been reported earlier(4). The animals were sacrificed 24 hours after infection and the numbers of staphylococcal colonies that could be recovered from their organs were determined by technics which have been described elsewhere(5). The results are presented in Table III.

Three of the animals which had received the largest amount of pertussis vaccine along with the sensitizing dose of bovine serum died acutely upon receiving the 1/2000 serum dilution used as diluent for the infective dose 27 days after sensitization. It is probable that this finding can be explained by the fact that the degree of allergic sensitization to bovine serum was the highest in animals that had received the largest amount of vaccine.

There was no evidence that the allergic shock had affected the response to infection, as no striking differences could be detected between the numbers of staphylococcal colonies recovered from the organs of animals sensitized with bovine serum alone and from animals having received in addition .006 or .0006 ml pertussis vaccine. While this experiment was in progress, however, the results of other unrelated studies disclosed that vaccination of mice with pertussis vaccine had a marked and lasting protective effect against infection with staphylococci. Evidence of this protective effect has been presented in a recent publication(6). Suffice it to state here that mice vaccinated with an amount of pertussis vaccine as small as 0.0006 ml (Sharp and Dohme preparation) still exhibit several weeks later a much increased resistance to staphylococcal infection. In view of this fact, it became evident that the protective effect of pertussis vaccine alone would have to be controlled in any study of the influence exerted by allergic shock on susceptibility to infection of animals sensitized with the help of this material as adjuvant. The experiment

TABLE IV. Effect of Allergic Shock on Fate of Staphylococci in the Organs of Mice.

Sensitizing treatment,* bovine serum (ml i.p.)	Time (days) between sensitizing and challenge	Challenge infection (i.v.): 0.1 ml staph. culture + 0.1 ml 1/1000 bovine serum											
		Nos. of staph. colonies† recovered from individual organs											
		Kidney				Liver				Lungs			
.5	10	4	14	85		27	39	103		13	22	31	
0	"	.2	1	12		53	140	148		.2	2	?	
.5	18	8	55	160	1900	24	210	310	1100	3	6	17	210
0	"	.1	1	5	35	5	48	56	230	0	0	.4	2
.5	23	560	3200	3500		78	170	300		0	1	4	
0	"	25	80	750		12	17	47		.1	1	1	

* All animals received .05 ml pertussis vaccine (Lederle) i.p.
† To be multiplied by 10^3 for whole lungs, and by 10^4 for whole liver and kidney.

presented in Table IV was planned with this requirement in mind.

It is seen that, whatever the time of infection, more colonies were recovered from the kidneys, livers, and lungs of animals that had been pretreated with bovine serum and with pertussis vaccine than from the organs of animals that had received the vaccine alone.

These findings suggest that the severity of the infectious process had been increased, at least temporarily, by the mild allergic shock resulting from the presence of 1/2000 bovine serum in the diluent used for injecting the bacterial suspension.

Summary. 1) Female albino mice of two different breeds were treated intraperitoneally with a mixture of bovine serum and pertussis vaccine. In confirmation of findings of other investigators, it was found that a large percentage of the animals so sensitized died within 30-60 minutes following the intravenous injection of very small amounts of serum 2-4 weeks after sensitization. The sensitizing effect appeared specific as no deaths occurred when either rabbit serum or egg albumin was used for the challenge injection. 2) Mice pretreated by intraperitoneal injection of bovine serum and pertussis vaccine, or of pertussis vaccine alone, were infected intravenously several weeks later with staphylococci resuspended in 1/2000 bovine serum. When they were sacrificed 24 hours after infection, it was found that many more staphylococcal colonies could be recovered from the kidneys, liver and lungs of the animals that had been sensitized to bovine serum, than from the organs of animals of the control group.

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Effect of Aspirin and Reserpine on Adrenocortical Response to Piromen in Man. (22751)

ROGER K. MCDONALD, VIRGINIA K. WEISE, AND RALPH E. PETERSON
N. I. of Mental Health and N. I. of Arthritis and Metabolic Diseases, Bethesda, Md.*

The elevated body temperature resulting from parenterally administered pyrogen in man is associated with a rise in the urinary output of 17-ketosteroids, corticosteroids, and an increase in concentration of free 17-hydroxycorticosteroids (17-OHCS) in plasma (1-3). Administration of aminopyrine has been shown to block the febrile response to typhoid-paratyphoid vaccine without altering the associated hemodynamic changes(4,5). Christy *et al.* have shown that aminopyrine also blocks the adrenocortical response to typhoid vaccine(6). Intravenously administered reserpine in dosages up to 1 mg/kg body weight failed to block the temperature elevation resulting from the injection of colon bacilli in rabbits(7). More recently Windle has noted a blocking effect of reserpine on fever production and eosinopenia following Piromen administration in monkeys (personal communication). Other studies in mice have reported that reserpine causes hypothermia (8).

The present study has been designed to elucidate the nature of the adrenocortical response to administration of a bacterial pyrogen, Piromen, and to observe the effects of aspirin and reserpine when given in conjunc-

* N.I.H., Public Health Service, U. S. Dept. of Health.

tion with Piromen. Specifically, test situations are aimed at clarifying the following points: 1. Is the increase in plasma free 17-OHCS concentration observed following Piromen due to increased adrenocortical secretion or decreased removal from the miscible pool? 2. Is hydrocortisone the adrenocortical secretion responsible for elevated plasma 17-OHCS levels following Piromen administration? 3. What is the effect of aspirin on the adrenocortical response to Piromen? 4. What is the effect of reserpine on the adrenocortical response to Piromen? 5. Is fever the cause of elevated 17-OHCS concentration resulting from the administration of Piromen?

Methods. Plasma free 17-OHCS were determined by the method of Silber and Porter (9), as modified by Peterson *et al.*(10). Hydrocortisone and corticosterone were determined specifically by isotope dilution(11,12). Eosinophil counts were made in a Speirs-Levy eosinophil counting slide. A. *Determination of Half-Life of Hydrocortisone.* Plasma half-life of hydrocortisone was determined in 4 young, normal subjects following intravenous administration of 0.3-0.4 μg Piromen/kg/body weight on one day and Piromen placebo on the other day. Three subjects received intravenous injections of 200 mg hydrocortisone sodium succinate and the 4th subject received 20-minute infusions of 200 mg free hydrocortisone in ethanol-water solution. Blood samples were withdrawn before administration of Piromen or Piromen placebo, immediately prior to hydrocortisone administration, and at appropriate intervals thereafter. Plasma 17-OHCS concentrations were determined for all specimens. B. *Determination of Miscible Pool and Turnover Rate of Hydrocortisone.* The miscible pool and turnover rate of hydrocortisone were determined on 1 normal, male subject, age 19, by the method of Peterson *et al.*(13). The study was carried out on each of 2 different days; 1 day the subject received 0.2 μg Piromen/kg body weight 2 hours prior to injection of hydrocortisone-4-C^{14}; on another day, he received Piromen placebo 2 hours before injection of hydrocortisone-4-C^{14}. C. *Effect of Aspirin and Reserpine on Plasma 17-OHCS Levels.* A total of 8 normal subjects, 4 males and 4 females, between 18 and 21 years of age, received in randomized order, saline infusion, Piromen, and Piromen plus aspirin. Observations made on days of saline infusions were used as control data. (The saline infusions do not alter normal diurnal fall in plasma 17-OHCS.) Three of the above subjects also received Piromen plus reserpine. Saline infusions were given from 8 to 10 A.M. at 3 ml/min. Piromen was administered intravenously (0.1 μg/kg body weight) at the beginning of each test (8 A.M.). Days on which the effect of Piromen, or Piromen plus reserpine, was determined, the subject was given 2 lactose capsules (identical in appearance to aspirin capsules) at 7 A.M., 9 A.M., 12 M., and 3 P.M. Days on which Piromen plus aspirin was tested, 2 capsules each containing 0.33 g of aspirin were given at same times. Subjects participating in the Piromen plus reserpine study were primed with oral reserpine in the following manner: first day, 0.25 mg reserpine q.i.d.; second day, 0.5 mg reserpine q.i.d.; third day, 0.75 mg reserpine q.i.d.; fourth and fifth days, 1.0 mg reserpine q.i.d. The test was carried out on the fifth day. The first blood sample was drawn immediately before administration of the test solution (8 A.M.). Blood was then withdrawn at hourly intervals until 12 M. and then at 2 P.M. and 4 P.M. Venous blood was obtained at 8 A.M. and 12 M. for eosinophil counts. Pulse and auscultatory blood pressure recordings were made hourly from 8 A.M. through 4 P.M. Oral temperatures were determined at 8 A.M., 10 A.M., 12 M., and 3 P.M. Fever is defined as an oral temperature above 37°C.

Results. Nature of Adrenocortical Secre-

TABLE I. Effect of Piromen on Concentration of Phenylhydrazine-Reacting Substances, Hydrocortisone and Corticosterone in Plasma.

		Phenylhydrazine method	Isotope dilution method
		μg/100 ml plasma	
Hydrocortisone	Control	15	15
	Piromen	24	25
Corticosterone	Control	—	1.8
	Piromen	—	3.0

TABLE II. Effect of Piromen on Hydrocortisone Half-Life.

Subject	Hydrocortisone preparation	Half-life (min.) Placebo	Piromen
DH	Sodium succinate	78	70
WG	*Idem*	113	110
WM	"	95	64
LS	Free	132	114
Avg		104	90

tion Following Piromen Administration. A comparison of the plasma concentration of 17-OHCS as determined by the phenylhydrazine method with the plasma concentration of hydrocortisone determined by the specific isotope dilution method shows that the concentration of 17-OHCS in the plasma during the control and the Piromen test is equal to the hydrocortisone concentration (Table I). Therefore, the Silber-Porter chromogens will be designated as hydrocortisone. By use of the isotope dilution procedure, it has also been shown that Piromen causes an increase in plasma corticosterone (Table I).

Miscible Pool and Turnover Rate of Hydrocortisone After Piromen. To ascertain whether the increase in plasma hydrocortisone is due primarily to decreased removal from the miscible pool or increased adrenocortical secretion, 2 tests were employed. First, the plasma half-life of hydrocortisone was determined approximately 3 hours following injection of Piromen or Piromen placebo. Table II indicates that the plasma half-life of hydrocortisone is not prolonged by prior administration of Piromen but rather is decreased by an average of 13%. Second, to determine more definitively if the increased plasma hydrocortisone concentration following Piromen administration was due to an increased secretion of hydrocortisone, the miscible pool and turnover rate of hydrocortisone were determined using hydrocortisone-4-C^{14}. Table III

TABLE III. Effect of Piromen on Miscible Pool and Turnover Rate of Hydrocortisone.

	μg % plasma F	Miscible pool, mg	Turnover rate, mg/hr
Control	14	1.5	.75
Piromen	35	7.6	4.70

presents the data in 1 normal subject on a control day and on a day he received Piromen. From this Table, it can be seen that the miscible pool was increased 5-fold and the rate of secretion of hydrocortisone was increased 6-fold.

Influence of Aspirin on Elevated Plasma Hydrocortisone Levels Observed With Piromen. Fig. 1 shows the mean hydrocortisone levels for 8 matched pairs receiving: 1) saline control infusion; 2) Piromen, and 3) Piromen plus aspirin. Intravenous injection of 0.1 μg Piromen/kg causes significant† increases in

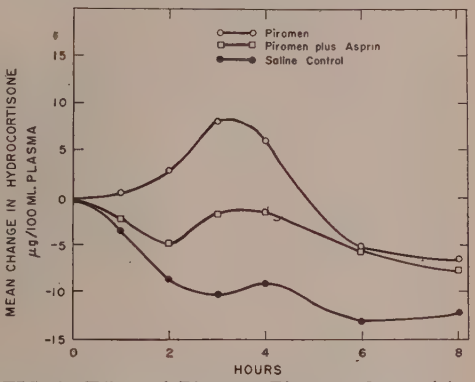


FIG. 1. Effect of Piromen, Piromen plus aspirin, and saline on plasma hydrocortisone levels. Individual points represent mean values for group of 8 normal subjects.

plasma hydrocortisone concentration for 8 hours following its administration. Hydrocortisone levels resulting from concomitant administration of Piromen and aspirin are significantly lower than those observed with Piromen alone at the 2nd and 3rd hours following the injection of Piromen. However, this dosage of aspirin is not sufficient to counteract completely the Piromen effect since there is a significant elevation of plasma hydrocortisone over saline control values at the 3rd through 6th hour of the test and probably a significant elevation at the 2nd and 8th hours ($P < 0.05$).

Influence of Reserpine on Elevated Plasma Hydrocortisone Levels Observed With Piro-

† The term "significant" is used to indicate statistical significance at the 1% level of confidence as determined by the t-test of Fisher.

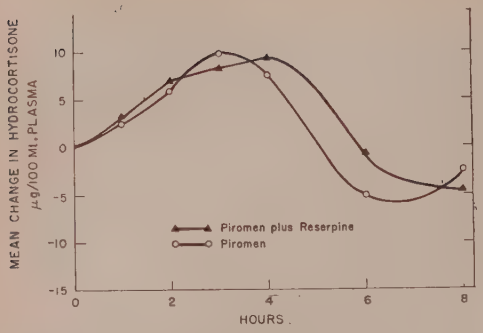


FIG. 2. Effect of Piromen and Piromen plus reserpine on plasma hydrocortisone levels. Individual points represent mean values for group of 3 normal subjects.

men. Fig. 2 shows the mean plasma hydrocortisone levels associated with Piromen and Piromen plus reserpine for 3 subjects. It is apparent that reserpine administered in this manner does not depress adrenocortical response to Piromen.

Observations on Temperature, Eosinophil Counts, Pulse, and Blood Pressure. Fig. 3 shows the temperature course during the different test procedures. Compared with the control observations, Piromen causes a significant elevation in temperature at 12 M. Aspirin administration causes a significant decrease in the temperature response to Piromen at 12 M. and 3 P.M. The 8 A.M. temperatures observed on 5 subjects reserpinized

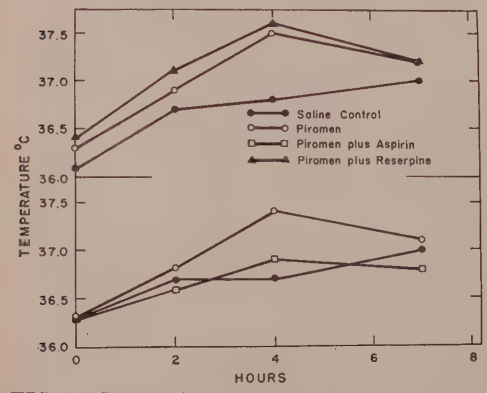


FIG. 3. Effect of saline, Piromen, Piromen plus aspirin, and Piromen plus reserpine on temperature. Upper graph represents mean values of 3 subjects. Lower graph represents mean values of 8 subjects.

in the manner described (the 3 subjects included in the adrenocortical evaluation and 2 additional) were not lower than the 8 A.M. temperatures on these same individuals on control and Piromen test days. Reserpine exerts no blocking effect on the temperature response to Piromen. The average percentage fall in the circulating eosinophils 4 hours after the beginning of the test was: saline, 39%; Piromen plus aspirin, 39%; Piromen, 58%; and Piromen plus reserpine, 57%. The 4-hour fall in the number of circulating eosinophils following Piromen is significantly greater than that following saline or Piromen plus aspirin. This dosage of Piromen causes a significant tachycardia from the 3rd through 7th hour of the test when compared with control values. Aspirin administration with the Piromen significantly lowers the pulse rates from the 4th through 8th hour. Reserpine causes bradycardia but the percentage increase in pulse rate following Piromen administration is not altered. No differences in the base-line blood pressure or blood pressure responses following Piromen or saline were noted between any experimental groups.

Relationship Between Body Temperature and Plasma Hydrocortisone Concentration. Analysis of individual relationship between plasma hydrocortisone concentrations and temperature rise 2 hours after Piromen administration indicates that only 1 of the 8 subjects revealed a temperature rise greater than 37°C. There is no significant difference in the mean 10 A.M. temperature between Piromen and control groups, while the plasma hydrocortisone levels are significantly higher with Piromen administration (mean difference is 12.9 μg%). Two hours after combined Piromen and aspirin administration, 2 of the 8 subjects showed a rise in plasma hydrocortisone (albeit less than they showed with Piromen alone) with a temperature less than 37°C. A 3rd subject manifested a temperature greater than 37°C, but there was no associated rise in the plasma hydrocortisone concentration.

Two hours after Piromen administration, 2 of the 3 subjects receiving reserpine manifested temperatures above 37°C, but all 3

subjects showed a rise in plasma hydrocortisone levels.

Comments. One of the observations of major interest in this study was the appearance of significantly increased adrenocortical secretory activity distinctly before the appearance of fever. Indeed, only one of the subjects receiving Piromen manifested an oral temperature above 37.6°C at the height of the temperature response. It is apparent that the effect of Piromen on adrenocortical activity is not dependent on fever production. It appears that the elevation of temperature and of plasma hydrocortisone levels are separate manifestations of the effect of Piromen. The inference of Christy *et al.*(6) that fever is the cause of increased plasma concentrations of 17-OHCS secondary to pyrogen administration is not supported by our observations.

The likelihood of salicylates in the dosages employed in this study causing a significant alteration in plasma 17-OHCS levels may be discounted on the basis of studies of Done *et al.*(14). These investigators were unable to show a significant rise or fall in plasma 17-OHCS levels associated with the administration of 1 gram of sodium salicylate administered hourly for 8 hours. Furthermore, Peterson and Bunim (unpublished observations) have shown that salicylates in a dose of 8 g/24 hrs exert no effect on the turnover rate of hydrocortisone in human subjects.

The difference in effect of reserpine on pyrogenic and eosinopenic effect of Piromen in monkeys observed by Windle (personal communication) and in our human subjects may be explainable by the dosages used. Windle employed a dose of 20 mg reserpine/kg body weight and a dose of 0.5-1 μg Piromen/kg body weight.

The studies on the plasma half-life of hydrocortisone and the miscible pool and turnover rate of hydrocortisone-4-C^{14} discount the possibility that factors other than increased adrenocortical secretion are responsible for the increased plasma 17-OHCS levels resulting from Piromen administration. It is apparent that the increased plasma hydrocortisone half-life which follows Piromen administration to anesthetized dogs(15) does not occur in unanesthetized humans.

Conclusions. 1. Intravenous administration of 0.1 μg Piromen/kg body weight to young, normal male and female subjects causes a significant rise in body temperature and plasma hydrocortisone levels. 2. Increased concentration of plasma free 17-OHCS following Piromen administration has been shown to be the result of increased secretion of hydrocortisone. 3. The rise in plasma hydrocortisone distinctly precedes the elevation of body temperature above 37°C. Fever is, therefore, not the cause of the elevated plasma hydrocortisone levels. 4. The increases in plasma hydrocortisone levels, eosinopenia, temperature, and pulse rate caused by Piromen administration are significantly lessened by the concomitant administration of aspirin. 5. Administration of reserpine in a dose of 4 mg/24-hr period caused no hypothermia in normal human subjects. 6. Pyrogenic, eosinopenic, and adrenocortical stimulatory effects of Piromen are not altered by the prior and concomitant administration of reserpine.

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Effect of Synthetic Lysine-Vasopressin on Plasma Hydrocortisone Levels in Man. (22752)

ROGER K. MCDONALD, VIRGINIA K. WEISE, AND RAYMOND W. PATRICK

National Institute of Mental Health, Bethesda, Md.*

Previous studies in this laboratory(1) have demonstrated that intravenous administration of highly purified arginine-vasopressin and highly purified lysine-vasopressin, as well as Pitressin, cause increased plasma hydrocortisone concentrations in man. Because of the possibility of ACTH-releasing or adrenotrophic substances contaminating these vasopressin preparations, a comparative study has been made of the effects of synthetic lysine-vasopressin and Pitressin on plasma hydrocortisone levels.

Methods. Five normal subjects, aged 18 to 21 years, were assessed for plasma hydrocortisone responses to intravenously administered Pitressin and synthetic lysine-vasopressin† in the following manner: At 8 a. m. on 2 different test days, each subject received intravenously 2 ml of vasopressin diluent (0.5% chlorobutanol solution adjusted to pH 3.6 with acetic acid). At 8:30 a. m., the subjects received intravenously on 1 test day 2 U. Pitressin and on the other test day, 2 U. of synthetic lysine-vasopressin. The order in which each subject received the 2 vasopressin preparations was randomized. Venous blood samples of 20 ml each were obtained through an indwelling Cournand needle prior to administration of the diluent (8 a. m.), 30 minutes following injection of diluent (*i.e.*, immediately preceding injection of the vasopressin preparation), and 30 minutes following the injection of vasopressin (9 a. m.). Plasma hydrocortisone concentrations were determined in duplicate on all samples by the method of Silber and Porter(2), as modified by Peterson *et al.*(3). Pulse rates and auscultatory blood pressures were determined at 1-minute intervals before, during, and after injection of Pitressin and synthetic lysine-vasopressin.

TABLE I. Effect of Pitressin and Synthetic Lysine-Vasopressin on Plasma Hydrocortisone Concentrations.

Time of blood withdrawal	Plasma hydrocortisone, μg/100 ml plasma	
	2 U. Pitressin	2 U. synthetic lysine-vasopressin
0′ (Pretest value)	16.6	18.0
30′ Following inj. of vasopressin diluent	14.3	16.6
30′ Following inj. of vasopressin preparation	25.7	25.6

Results. Table I shows the mean plasma hydrocortisone‡ concentrations for the 5 subjects on the Pitressin test day and on the synthetic lysine-vasopressin test day. There is no significant difference between the plasma levels elicited by 2 U. of Pitressin and 2 U. of synthetic lysine-vasopressin. The mean blood pressure response to both vasopressin preparations was the same (approx. 20 mm Hg increase) while the pulse rates were un-

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‡ It has been shown in other studies(4,5) that the Silber-Porter chromogens are equivalent in concentration to free hydrocortisone.

affected by either.

Summary. 1. In man, the intravenous injection of synthetic lysine-vasopressin causes a rise in plasma hydrocortisone concentration comparable to that following the intravenous administration of Pitressin. The effect of the latter is therefore not due to impurities. 2. The pressor effect of synthetic vasopressin is of the same order as that noted following the injection of Pitressin.

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Some Effects of Parathyroid Extract and Cortisone on Metabolism of Strontium and Calcium.* (22753)

JAMES A. BACON, HOMER PATRICK, AND SAM L. HANSARD
(Introduced by B. L. O'Dell)
University of Tennessee-Atomic Energy Commission Agricultural Research Program, Oak Ridge, Tenn.

Diuresis with an increased urinary excretion of calcium and phosphorus results when parathyroid extract is administered intraperitoneally to rats(1,2). After injection of the hormone into normal animals the blood calcium rises followed by an accompanying rise in urinary calcium. Burrows(3) reported that the rise of blood calcium following injection of parathyroid extract is a result of mobilization of calcium from bones. The subepiphyseal plate appears to be the region of the bone from which the parathyroid extract brings about removal of calcium(4). The kidneys of rats receiving parathyroid extract contained 365 times more calcium than the controls when observed within 24 hours after receiving the extract containing the hormone. However, after the first 24 hours no significant difference was observed between treatments. Cortisone has been found to increase excretion of potassium, sodium and chloride in normal dogs. Its relation to calcium deposition in soft tissues and bone growth(5,6) has been reported from several laboratories.

The investigation here reported was undertaken to determine whether strontium would respond similarly to calcium when experimental animals receive parathyroid extract and also whether cortisone would counteract deposition of these minerals in kidney tissue in rats receiving parathyroid extract.

Methods. Two experiments were conducted during these studies. Uniform Rockland rats weighing approximately 160 g were used for these experiments. The rats were housed in metabolism cages for ease of making balance studies as outlined by Hansard and Comar (7). In each experiment the rats were given 5 μc of calcium-45 or strontium-89, subcutaneously 24 hours before receiving injections of the hormones. The parathyroid extract was administered subcutaneously in doses of 100 I. U. each, 24, 32, 48 and 56 hours after receiving the nuclide. When cortisone was administered, it was given in 4 subcutaneous injections of 25 mg in a saline solution, at the same time as the parathyroid extract. Sixteen hours after receiving the final hormone

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TABLE I. Relation of Parathyroid Extract to Calcium and Strontium Metabolism.* Six rats per series.

Treatment	Nuclide	Femur, %/femur	Femur, %/mg Ca.	Urinary excretion, %/rat 24 hr	48 hr	72 hr	Kidney, %/g (wet wt)	Fecal excretion, %/rat 24 hr	48 hr	72 hr
None	Sr-89	4.2	.035	5.3	1.36	.43	.02	5.9	4.3	1.24
PTH	"	4.7	.041	5.4	1.11	.74	.26	6.5	5.0	.94
None	Ca-45	4.7	.034	1.7	.24	.13	.00	5.3	2.3	.99
PTH	"	3.6	.022	2.9	.30	.59	.34	5.2	2.5	.78

* Expressed as % of administered dose.

injection the rats were sacrificed and the femur and kidneys assayed for calcium-45 or strontium-89. The daily urine and feces were collected and assayed for the radionuclide being studied. All samples were dry-ashed at 650°C, dissolved in concentrated hydrochloric acid and an aliquot dried in a stainless steel counting cup and counted under a Geiger-Muller tube. Total calcium was determined by precipitating calcium as oxalate and titrating the precipitate with potassium permanganate using the method described by Comar *et al.*(8).

Results. Since the rat uses calcium in preference to strontium, the first experiment was designed to study the influence of parathyroid extract on both calcium and strontium metabolism and this relationship. The results are summarized in Table I. Parathyroid extract influenced the metabolism of strontium and calcium similarly as related to deposition in kidney tissue and urinary excretion. Statistical analysis of the data indicated a significant difference at the 5% level of confidence between treatments in 72 hour urinary excretion and kidney tissue deposition. Parathyroid extract caused diuresis, increased urinary excretion of calcium and strontium during 24 to 48-hour period after administration of the hormone and increased deposition of these nuclides in the kidneys. The amount removed from the bones was not of sufficient magnitude to detect differences by analysis of bones or autoradiographs.

In the second experiment the relation of cortisone to the influence exerted by parathyroid extract on calcium and strontium metabolism was studied. Parathyroid extract, alone or with cortisone, significantly (5% level) increased urinary excretion of calcium and strontium during the 24 to 48-hour period after administration of the hormone over the control rats. The significant increase in calcium and strontium deposition in the kidney tissues observed when parathyroid extract was administered did not appear when cortisone was administered with parathyroid extract. Cortisone prevented the accumulation of both calcium and strontium in kidney tissue. It is interesting to observe that whereas parathyroid extract increases both urinary and kidney calcium and strontium; cortisone influences

TABLE II. Relation of Parathyroid Extract and Cortisone to Calcium and Strontium Metabolism.*

Treatment	No. rats	Nuclide	Femur, %/femur	Urinary excretion, %/rat 24 hr	48 hr	72 hr	Kidney, %/g (wet wt)	Fecal excretion, %/rat 24 hr	48 hr	72 hr
None	4	Sr-89	3.2	6.2	1.13	.61	.014	8.5	4.12	2.28
PTH	6	"	3.1	6.9	1.61	1.45	.390	8.4	4.23	1.07
PTH + cortisone	6	"	2.9	8.8	1.48	1.50	.014	8.6	5.09	1.06
None	4	Ca-45	1.9	1.6	.21	.09	.037	1.8	.71	.44
PTH	6	"	2.1	1.5	.19	.22	.166	2.0	.68	.33
PTH + cortisone	6	"	2.1	2.1	.36	.27	.032	2.2	.97	.43

* Expressed as % of administered dose.

only the accumulation in the kidney tissue. Cortisone did not influence the diuresis caused by the parathyroid extract.

Summary. 1) Parathyroid extract increased the urinary excretion of calcium and strontium as well as increased deposition of these minerals in the kidney tissue. 2) Cortisone prevents accumulation of strontium and calcium in kidney tissue but does not influence urinary excretion or diuresis.

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Deoxyribonucleic Acid Content and Mitotic Activity of Vagino-Cervical Epithelium in Colchicine-Treated Mice During Estrus.* (22754)

Dante G. Scarpelli† (Introduced by E. von Haam)

Department of Pathology, Ohio State University, Columbus

The estrous cycle of the mouse is characterized by alternating periods of rapid cell growth and involution of genital tissues and affords an excellent opportunity for study of fundamental chemical aspects of cellular growth in response to hormonal stimulation. Quantitative cytochemical studies of estrus have been limited largely to the study of resting cells of uterine glands, to surface epithelial cells of rat uteri(1) and to total nucleic acid content of uteri. Greater accuracy of microspectrophotometric analysis of individual cells in elucidating their chemical status over chemical analysis of tissues containing heterogeneous cell types has been shown. The value of exfoliative cytology in following physiological and pathological events in the female genital tract has been well established(3-5). The present work was undertaken to study the deoxyribonucleic acid (DNA) content of exfoliated cervical and vaginal cells during the estrous cycle and to correlate these findings with DNA content and mitotic index of basal cells in tissue sections of vaginal fornix.

Materials and methods. Fourteen female C3H mice, 2 to 3 months of age and weighing 18 to 22 g, were divided into 6 groups. Groups 1 and 2 consisted of 3 animals each, Groups 3, 4, 5 and 6 of 2 animals each. Animals of Groups 5 and 6 were ovariectomized 10 days before start of experiment. Animals were kept in air-conditioned room (68-72° F), fed Purina Dog Checkers supplemented by daily addition of fresh bread and carrots. Daily vaginal smears were taken and animals sacrificed at various stages of estrous cycle. Animals of Group 6 were injected subcutaneously with 25 RU of theelin in peanut oil and sacrificed 10 and 20 hours later. Animals of Group 5 served as ovariectomized controls. Mitoses in basal cells of vagina and cervix were arrested in metaphase by the colchicine method of Bullough(6) in which period of treatment was limited to 5 hours to prevent interference with normal mitotic rate. Each animal received 0.1 mg of drug in 0.25 ml of normal saline subcutaneously and sacrificed by cervical dislocation 5 hours later. Smears were prepared immediately and stained by method of Papanicolaou(7). Vaginas and uteri were removed *in toto*, fixed in 10% buf-

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† National Science Foundation Postdoctoral Fellow in Pathology.

TABLE I. Nuclear DNA of 585 Nuclei and Mitotic Index of Basal Cells in Various Stages of Estrus following Colchicine Treatment, 5 Yr.

Group	No. of animals	Stage of estrus	Total nuclei counted	Diploid cells %	Diploid cells DNA, rel. units	% intermediate cells	Tetraploid cells %	Tetraploid cells DNA, rel. units	Mitotic index
1	3	Proestrus	150	82	1.03 ± .09	12.6	5.3	1.98 ± .21	40.2 ± .41
2	3	Estrus	85	67.3	1.07 ± .11	22.2	10.5	2.04 ± .30	223.8 ±10.2
			20*	100	1.01 ± .27	0	0		
			15†		.53 ± .31	0			
3	2	Metestrus	80	100	1.11 ± .22	0	0		9.5 ± 1.11
4	2	Diestrus	75	98.7	1.10 ± .16	1	0		7.3 ± .92
5	2	Castrates	75	100	1.08 ± .13	0	0		11.8 ± 1.23
6	1	Castrate + theelin, 10 hr	45	71	1.05 ± .17	22.2	6.7	2.10 ± .18	28.7 ± 3.2
	1	Castrate + theelin, 20 hr	50	64	1.06 ± .11	26	10	1.90 ± .22	48.3 ± 6.4

* Pyknotic nuclei. † Advanced pyknotic nuclei.

fered formalin, embedded in paraffin and sagittal serial sections cut at 7 and 15 μ thickness. 7 μ sections were stained with hematoxylin and eosin for mitotic counts. Sections cut at 15 μ were used for demonstration of DNA. They were subjected to the Feulgen reaction(8) carried out on smears and tissue sections after hydrolysis with N HCl at 60°C for exactly 12 minutes. Intact interphase basal, parabasal and keratinized cell nuclei selected at random in each vaginal smear stained by the Feulgen method were measured by a microspectrophotometer similar in design to that described by Pollister(9) and satisfying the requirements set by Pollister and Ornstein(10). A wavelength of 560 mμ was used, supplied by Bausch and Lomb grating monochromator. This wavelength represents maximum absorption peak of stain. To minimize light scattering, slides of smears were mounted in oil with a refractive index of 1.572, which matched that of unstained mucus. Relative amounts of DNA of individual nuclei in smears were obtained by multiplying extinction values by the nuclear area since all the nuclei were flattened(11). The major (a) and minor (b) axes of nuclei were measured with an optical micrometer and the area determined by the formula $A = \pi \frac{1}{2}a \times \frac{1}{2}b$. To include basal cells from both ectocervical and vaginal epithelium interphase basal cell nuclei were measured in cervical and vaginal portions of the vaginal fornix. Since care was taken to select uncut nuclei with no overlap, this sharply limited the number of nuclei measured, as cells were crowded especially during estrus. Relative amounts of nuclear DNA in tissue sections were obtained by the method of Swift(12).

Results. The results of DNA measurements in 550 basal and parabasal cell nuclei, 20 keratinized cells containing pyknotic nuclei, and 15 keratinized cells showing advanced pyknosis, karyolysis or karyorrhexis are presented in Table I. Representative histograms were prepared showing the DNA content of basal, parabasal and keratinized cells during various stages of estrus. Basal cells containing intermediate and tetraploid amounts of nuclear DNA were in vaginal smears and tissue sections during early proestrus, where keratinization was not of sufficient thickness to impair basal cell exfoliation. During estrus basal cells containing increased nuclear DNA, *i.e.* intermediates and tetraploids, were absent in vaginal smears while a considerable number were present in the tissues. Twenty hours following subcutaneous injection of theelin in ovariectomized mice the number of exfoliated basal cells containing increased nuclear DNA in smears had decreased, while basal cells containing increased nuclear DNA in the tissues had increased. Appearance of cells showing increased amounts of DNA paralleled the increased mitotic activity of vaginal and cervi-

cal basal cells during proestrus and estrus. During periods of quiescence (metestrus, diestrus and castrates), when mitotic activity was low, a constant diploid value of nuclear DNA was present (Table I). Even though some of the superficial cells exfoliated during estrus showed a marked degree of nuclear enlargement and early keratinization, the DNA content remained at a diploid value. A significant decrease in nuclear DNA occurred only in keratinized cells showing advanced pyknosis, karyolysis or karyorrhexis (Fig. 2).

Discussion. The stimulating effect of estrogenic hormones on growth of genital epithelium is well established, although the bulk of uterine growth following estrogen treatment is largely due to increase in cell volume rather than to cell multiplication(13). Allen *et al.*(14) utilizing colchicine to arrest mitoses have shown that estrogens actually markedly increase the rate of mitosis in vaginal and cervical epithelium of the mouse. The presence of numerous mitoses and interphase basal cells containing intermediate and tetraploid values of nuclear DNA in vaginal and cervical tissue during proestrus and estrus is in accord with this observation. This is in contrast to previous reports of constancy of nuclear DNA in hormonally stimulated uteri (1). However, several reasons may account for this difference. First, cells of vaginal and cervical epithelium where highest mitotic activity is seen, were not studied; secondly, due to the fact that vaginal and cervical epithelium represents but a small fraction of total uterine weights, a chemical determination of nucleic acids of the total uterus does not reflect the high degree of DNA synthesis occurring there. Bloch(15) found that although colchicine arrests cells in mitosis it does not interfere with DNA synthesis and that nuclei falling into higher DNA classes began to appear 10 hours after colchicine treatment. The period of colchicine treatment used in this study was of insufficient length to impair the rate of mitosis or to result in polyploidy due to DNA synthesis without subsequent mitotic division. The increase in nuclear DNA is probably due to a premitotic synthesis of DNA, as has been shown to occur in rapidly growing tissues by Patau and Swift(16). Furthermore, since Howard and Pelc(17) found that synthesis of DNA in the interphase nucleus occurs within a relatively short time is completed some time prior to mitosis, it is not surprising to find resting basal cells with increased amounts of nuclear DNA during periods of augmented growth. The preponderance of basal cells showing intermediate DNA values, and the relatively small tetraploid population, may indicate that once DNA synthesis has been completed division quickly ensues. This may explain the low mitotic index of uterine tissue following hormonal stimulation reported by Drasher and Salvatore(2,13). Basal cells containing increased amounts of DNA were present in vaginal smears taken during proestrus. The paucity of basal cells in smears during late proestrus and throughout estrus is due to the thick layer of keratin which prevents their exfoliation and is not a true reflection of the high degree of DNA synthesis present in the tissues during these periods.

Although cells exfoliated into the vaginal pool are more prone to cellular death and pyknosis, microspectrophotometric studies revealed that the majority of nuclei contained the normal diploid amount of DNA. This was true even though nuclear size was increased and early keratinized cells showing advanced pyknosis. This is in agreement with Leuchtenberger's report(18) on nuclear DNA changes during pyknosis.

It would appear from these experiments that the DNA content of exfoliated basal and parabasal cells in the vaginal pool is quite stable and within limits, corresponds to the DNA content of these cells in the cervical and vaginal mucosa during various physiological states. These findings indicate that except during high estrus it is possible to study DNA synthesis of epithelial cells during the estrous cycle by microspectrophotometric analysis of representative vaginal smears.

Summary. 1. Increased amounts of nuclear DNA were found in basal cells during periods of augmented growth in the estrous cycle. 2. These changes in nuclear DNA parallel the mitotic activity of the basal cells. 3. Exfoliated basal cells containing intermediate

and tetraploid amounts of DNA were found only during early proestrus. 4. The paucity of a tetraploid nuclear population during estrogenic stimulation may indicate rapid cellular division once DNA synthesis has occurred.

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Bioenergetic Basis of Light-induced Fat Deposition in the White-crowned Sparrow.* (22755)

JAMES R. KING† AND DONALD S. FARNER
Laboratories of Zoophysiology, Department of Zoology, State College of Washington

It has been clearly established in at least 6 species of migratory birds that vernal premigratory deposition of fat in visceral and subcutaneous depots can be simulated by subjecting such birds to artificially prolonged daily photoperiods(1-6). That this phenomenon is the consequence of temporary hyperphagia and not the result of increased time for feeding, as daylength increases, appears evident from the experiments of Odum and Major(6) with white-throated sparrows (*Zonotrichia albicollis*) and Koch and de Bont(5) with a single chaffinch (*Fringilla coelebs*). These investigators, and Wallgren(7) also, have proposed that this response must involve the hypothalamus, a suggestion which is consistent with known effects of light on avian hypothalamo-hypophyseal system(8) and the role of hypothalamic subcenters in regulation of food intake in laboratory mammals(9-11). This investigation provides a more extensive and more quantitative basis for understanding this interesting phenomenon by *1.* definition of temporal course of fat deposition in wild populations of a typical migratory passerine bird (*Zonotrichia leucophrys gambelii*), *2.* measurement of energy intake during premigratory period in captive birds under natural conditions of light and temperature, and *3.* examination of bioenergetics of fat deposition obtained experimentally with prolonged daily photoperiods.

Material and methods. Animals: White-crowned sparrows (*Z. l. gambelii*) were captured from migrant and wintering populations in the vicinity of Pullman, Wash. Specimens for extraction of fat were weighed and killed immediately and kept frozen until extractions

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† National Science Foundation Predoctoral Fellow.

were made. Reserve stocks were maintained in large outdoor flight cages. These, as well as experimental birds, were fed a nutritionally adequate chick-starter mash having fat, protein, and carbohydrate components of 3.1%, 20.7% and 74.7% respectively, plus mineral and vitamin additives. The two batches had caloric contents of 4.439 and 4.552 kcal/g, respectively. *Fat determinations:* Entire carcasses were quantitatively extracted with diethyl ether in Soxhlet apparatus(15). Data are expressed as grams of ether-extractable lipid/100 g live body weight; this ratio is designated as *lipid index.* In all, 110 carcasses were extracted from collections in January to May, 1952, 1953, and 1954. The 1953 series of lipid indices, for the greater part, was obtained in conjunction with studies of morphology and composition of subcutaneous fat bodies(15,16). *Energy intake:* Birds were housed individually in Hendryx breeding cages (22 × 41 × 26 cm) from which floors had been removed. Each cage was placed in a close-fitting pan, 12.5 cm in depth, which permitted complete collection of excreta and spilled food. Food weighed to nearest 0.1 g was provided daily in hopper attached to side of cage; the unused feed from previous day was also weighed. Feed and water were continuously available. Moisture content of feed was determined weekly. Periodically (intervals of 4 to 7 days), pans were changed at a fixed hour. Spilled food and excreta were separated and dried to constant weight at 98° C. From these data the *gross energy* intake was calculated. Subtracting from this the total caloric value of excreta collected during the same period gives the *metabolizable energy*(14,17), which, except for energy potentially lost in specific dynamic action of food ingested and in bacterial fermentation, is the energy actually available to the bird. Calorimetric determinations of food and excreta were made with oxygen bomb calorimeter with experimental error of less than 0.5% between duplicate samples. Experimental error in de-

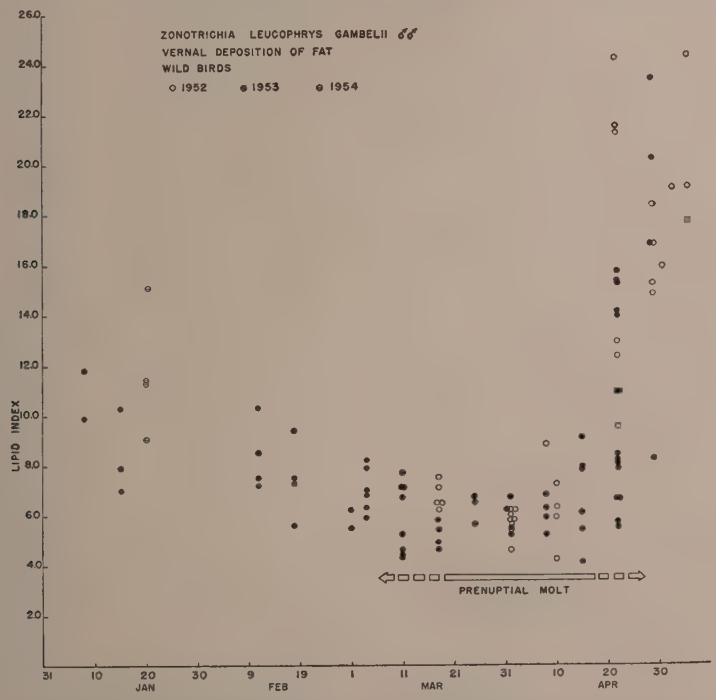


FIG. 1. Lipid indices in wild populations during late winter and spring. Migratory movement began during last 10 days of April.

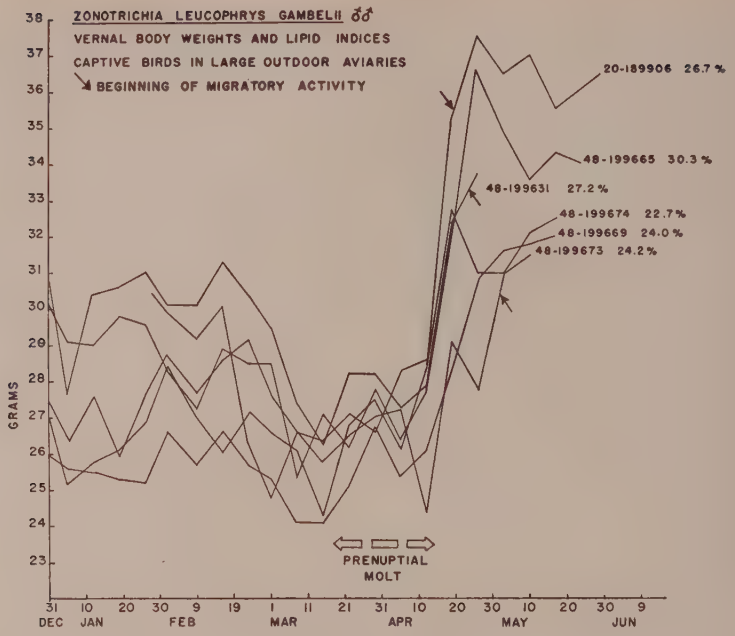


FIG. 2. Percentages at right are terminal lipid indices. Oblique arrows indicate onset of *Zugunruhe* in 3 birds in which activity was recorded.

termination of metabolizable energy is of the order of 3%. *Respiratory metabolism:* Oxygen consumption and carbon dioxide production were measured with apparatus similar to that of Schwabe and Griffeth(18). Attainment of *quasi*-basal conditions was guided by precautions suggested by Benedict(19) and Wallgren(7). *Activity recording:* Recording was by scheme similar to that described by Farner and Mewaldt(20) with the addition of impulse-counting circuits, which gave a numerical total for each hour for each cage. It must be emphasized that activity recording system of this type does not measure absolute or total activity but gives an *index of activity* as a fraction of total activity of a given subject. Since this fraction may differ among experimental animals, activity levels must be compared on a relative basis(21).

Results. Vernal Fat Deposition: Fig. 1 shows eruptive nature of the vernal premigratory fat deposition in nature. Migratory movement began during the last week of April in each of the 3 years. This pattern is similar to premigratory fat deposition for *Z. albicollis* in Georgia(22), except that the amount of fat deposited appears to be somewhat greater in *Z. l. gambelii*. This difference, however, might be only the result of differences in opportunity for collecting specimens during the first day or two of migratory movement. Odum and Connell(23) have recently shown that lipid indices of 25 to 30% are common in passerine birds captured in migration.

Fig. 2 demonstrates that the pattern of premigratory fat deposition, as indicated by a sample from the wild population, is substantially the same for individual birds held under natural conditions of temperature and photoperiod. Here it has been necessary to use body weight as an index of fat deposition. This is justifiable since our data and those of Odum and Perkinson(22) indicate an annual variation in fat-free weight of less than 10%. Therefore, variation in body weight of birds routinely weighed at fixed times of the day is due largely to variation in fat content. The terminal lipid indices in these captive birds are slightly higher than those of wild birds,

probably because of somewhat restricted activity and readily available food supply. However, the date of beginning and rate of deposition of premigratory fat as well as temporal relationship with prenuptial molt are essentially the same. *Experiment 1:* The intake of metabolizable energy, the daily activity, and the variation in body weight were measured over 7-day intervals in 6 males confined in outdoor cages during the late winter and spring of 1955. It was found that a marked increase in body weight (fat deposition) in the last half of April was accompanied by a definite increase in energy intake. In 4 birds for which these data are acceptable, the increase was 30 to 50% above the preceding minimum. Although the energy *utilization coefficient* (metabolizable energy/gross energy $\times$ 100) increased in all birds from an average of approximately 70 in March to 76 in early June, the change was continuous and gradual and could not logically explain the very abrupt increase in metabolizable energy income. Likewise, ambient temperature oscillated only slightly around an increasing mean, with no extreme minima in April which can be correlated with timing or magnitude of maximum energy intake. Daylength, and consequently time for feeding, increased 9% (14.4 to 15.7 hours) during the last 3 weeks of April. A normal prenuptial molt occurred. The daily activity of birds continuously increased during the experiment, as previously shown(21) with *Z. l. gambelii* under the same conditions. Pronounced nocturnal unrest, or *Zugunruhe* (the behavioral homologue of migratory movement in caged migrants during migratory season)(24) developed on 28 April. *Exp. 2:* In mid-January, well after annual period of refractoriness to photic stimulation (25,26), 8 males were placed in individual metabolism-activity cages in constant-condition room maintained at 12 $\pm$ 2°C and 35-40% relative humidity. For 29 days these birds received 9 hours light per day (0900-1800) from incandescent lamps so arranged that light intensity measured at floor of cages was 35-40 foot-candles. A photoperiod of this duration is essentially subliminal for stimulation of testicular development and fat deposition. On Feb. 13 the group was divided into 2 groups of 4 each. One group was retained as untreated control. The experimental group was transferred to another room maintained at same temperature, humidity, and light intensity. The photoperiod for this group was then increased by 2-hour daily increments to 20 hours per day (lights out 1900-2300). The increase in photoperiod evoked patterns of hyperphagia and concomitant increase in body weight (fat deposition) which were very similar to those observed under natural conditions. A temporary increase in energy income of 27 to 43% above preceding minima occurred. The mean utilization coefficients in both groups were relatively constant at about 71 throughout experiment. The mean terminal lipid indices for experimental and control birds were 23.5 ($\sigma = 3.05$) and 12.3 ($\sigma = 3.34$) respectively. The difference is statistically significant ($P = 0.03$). Standard metabolic rates of controls and experimentals, computed on basis of lean body weight, show a sharp divergence after beginning of the increase in photoperiod, the experimentals uniformly attaining a rate approximately double that of controls. Respiratory quotients in the 2 groups ranged between 0.72 and 0.82. While it is very probable that this *apparent* increase in standard metabolic rate is due in part to a decrease in depth of sleep, as Bergman(29) has shown to occur in several passerine species during development of *Zugunruhe*, it is of particular importance here that there was no *decrease* in standard metabolic rate which could contribute energy to fat deposition. This aspect of our investigations will be considered in detail in subsequent publication. Activity of the 2 groups shows that there is no *decrease* in muscular activity of the 20-hour experimentals which could spare energy for fat deposition. By the end of the experiment all of experimentals were definitely in *Zugunruhe*. A very light molt occurred only in the spinal tract after the change to the 20-hour photoperiod. No molt was observed in the 9-hour controls.

Discussion. These observations suggest very strongly that the premigratory fat depo-

sition in *Z. l. gambelii* is largely, if not entirely, the result of a photically induced hyperphagia. It is evident at least in the white-crowned sparrow, in the chaffinch(5), and the Ortolan bunting (*Emberiza hortulana*)(7) that premigratory adiposity cannot result from temporary decline in basal energy requirements as Groebbels(12) has suggested, nor from a combination of this and a moderate increase in food intake, as proposed by Merkel(13). Also it is apparent that there is no decrease in muscular activity nor increase in digestive efficiency of sufficient magnitude to account for the surplus of energy stored as fat. Our data apparently are inconsistent also with the hypothesis(14,24,27,28) that "productive" or surplus energy available for fat deposition is the result *primarily* of energy-sparing effects of increasing environmental temperature in spring, and the cessation of energy-demanding prenuptial molt plus increased energy intake made possible by more daylight for feeding. It seems unlikely that the eruptive nature and temporal precision of vernal fat deposition can be dependent to any substantial degree on such a fluctuating variable as environmental temperature, although it may have a secondary modifying role. With respect to the effect of increasing daylength, data from Exp. 1 show an average increase of 36% in rate of caloric intake (1.54 to 2.10 kcal/bird/hr of daylight) during a period when daylength increases by only 9%. Clearly there is no simple, linear relationship. The data also indicate that neither elevated environmental temperatures nor cessation of molt are essential in development of vernal adiposity. The simultaneous occurrence of molt and fat deposition has been noted previously under experimental conditions by Farner and Mewaldt(26) and Odum and Major(6).

In view of the similarity between development of obesity in mammals with hypothalamic lesions(11) and of premigratory fat deposition in birds it appears probable, as Koch and de Bont(5) have suggested, that this photically induced hyperphagia in birds is mediated by hypothalamic feeding centers. Although this hypothesis awaits the demonstration of feeding centers in the avian hypothalamus, such a mechanism controlled by vernal increase in daily photoperiod is consistent with observed annual precision of premigratory fattening.

Summary. In *Zonotrichia leucophrys gambelii* there is a striking deposition of fat in subcutaneous and visceral depots following prenuptial molt and preceding beginning of vernal migration. During the last 2 weeks of April, in natural populations, mean lipid indices increase from about 5% to more than 20%. A similar premigratory fat deposition occurs in captive birds under natural conditions of photoperiod and temperature. Similarly in these captive birds this fat deposition is followed by development of migratory behavior (*Zugunruhe*). Measurements of metabolizable energy intake in these birds show that fat deposition is accompanied by a marked increase in energy intake to levels of 30 to 50% above the preceding minima. This temporary hyperphagia represents an altered physiologic state rather than simply the effect of more daylight for feeding since, during hyperphagia, the amount of daylight increases only 9%. The temporary hyperphagia is the principal factor responsible for positive energy balance required for premigratory fat deposition. The role of daily photoperiod in development of temporary hyperphagia was demonstrated experimentally by subjecting birds in mid-winter to 20 hours of light per day. These developed a hyperphagia and an accompanying fat deposition very similar to those observed in spring under natural conditions.

We wish to acknowledge the assistance of Dr. L. R. Mewaldt in obtaining some of the lipid indices reported herein.

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Glycogen Deposition in Squamous Metaplastic Epithelium of the Rat Uterus.* (22756)

WALTER J. BO (Introduced by C. J. Hamre)
Department of Anatomy, University of North Dakota School of Medicine, Grand Forks

The deposition of glycogen in the normal uterus of the rat has been studied by both chemical(1) and histochemical methods(2). It has been demonstrated by chemical analysis that the total glycogen content of the rat uterus varies with the stage of the estrous cycle, being maximal during proestrus and minimal during diestrus. Histochemically, it has recently been shown that the glycogen of the rat uterus is limited to the muscle cells of the myometrium; in the proestrous animal the material is abundant throughout the longitudinal layer but is irregularly distributed in the circular layer while in the diestrous uterus, the glycogen content of the longitudinal muscle layer is greatly reduced and that of the circular muscle layer has disappeared entirely.

The distribution of glycogen in abnormal uteri, particularly in epithelial metaplasia of the rat uterus has not been reported. One of the methods used to produce and study uterine metaplasia in the intact rat is by placing the animal on a vit. A deficient diet(3). Using this method to produce epithelial metaplasia, the present investigation was undertaken to study, by the periodic acid-leucofuchsin technic, the distribution of glycogen in the metaplastic uterine glands of the intact rat.

Materials and methods. Twenty-five rats of the Wistar strain were used in these experiments. The animals were 20 to 22 days of age and weighed approximately 35 to 40 g when placed on the vit. A free diet.† The rats were killed with chloroform during the 9th to 14th week of the deficiency and the uteri were fixed immediately. A portion of each uterus

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† Vitamin A Test Diet U.S.P., Nutritional Biochemicals Corp., Cleveland, O.

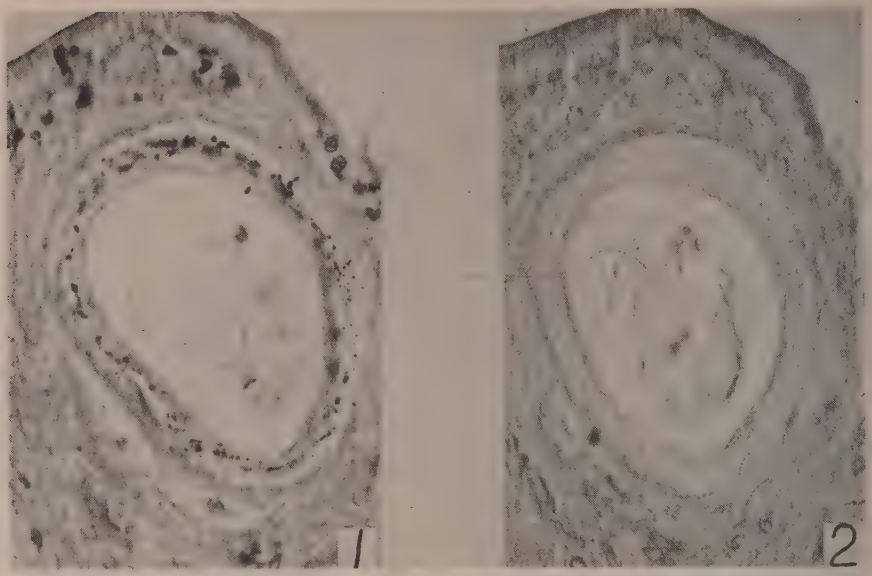


FIG. 1. Photomicrograph of a metaplastic gland following vit. A deficiency. The section was stained by the periodic acid-leucofuchsin method. Note dark glycogen granules in epithelium of metaplastic gland. × 410.

FIG. 2. Photomicrograph of a control section, close to that illustrated in Fig. 1, which was incubated in malt diastase to remove glycogen before staining. Note absence of glycogen granules from epithelium of metaplastic gland. Residual color in the section is due to staining of mucopolysaccharides and glycoprotein. × 410.

was fixed in cold modified PAF (10% formalin in 80% ethyl alcohol saturated with picric acid), cold acetone and in 10% formalin containing 2% calcium acetate. The present report is concerned with the study of glycogen deposition in epithelial metaplasia of the uterus following vit. A deficiency. After fixation in PAF the tissue was dehydrated, embedded in paraffin and sections were cut 12 μ in thickness. These were treated with periodic acid and then stained with leucofuchsin (4). Both glycogen and mucopolysaccharides are stained a reddish purple. In order to distinguish between them, control slides were incubated for an hour in 1% malt diastase buffered at pH 6.8 to remove the glycogen before applying the periodic acid-leucofuchsin procedure. No counterstain was used.

Observations. In the metaplastic glands that were observed in the uteri of the vit. A deficient animals, PAS positive material was present in the cells of the stratified epithelium. The granules were present primarily in the intermediate layer of the epithelium. In the control sections that were treated with 1% malt diastase, the material was not present indicating the presence of glycogen (Fig. 1 and 2).

The secretory product of the luminal and glandular epithelium that did not show any metaplastic changes was PAS positive but was resistant to 1% malt diastase which indicated that the material was not glycogen but a mucopolysaccharide.

The amount of glycogen observed in the tunica muscularis was variable; none was observed in the majority of the animals. In some of the animals only a trace of glycogen was observed in the longitudinal layer. The distribution of the glycogen in the uterine muscle was similar to that previously reported in the castrate and diestrous rat(2).

Discussion. The normal epithelium of the rat uterus, as was reported previously(2), does not contain any glycogen. However, the present results show that in the metaplastic epithelium following vit. A deficiency, glycogen granules are present in the cells of the

new epithelium.

The significance of glycogen deposition in squamous metaplasia of the uterus following vit. A deficiency and the biochemical enzyme systems involved is not known at the present time. There is evidence that the most important single factor which determines the rate of mitosis in tissue, such as mammalian epidermis, is the energy supply within the cells which depends upon the intracellular availability of glucose derivatives(5). If it can be assumed that because of increased mitotic activity the energy requirements of metaplastic epithelial cells is greater than for normal uterine epithelial cells, then the glycogen present in the metaplastic cells may be a source of readily utilizable energy.

The present results on glycogen deposition in epithelial metaplasia differs from the observation made in the human(6). In one instance a single squamous metaplastic cervical gland of one patient was reported to be free of glycogen. Since there appears to be a difference in glycogen deposition in squamous metaplasia, investigations are under way to determine whether the deposition of glycogen in stratified squamous metaplasia is peculiar to the changes following vit. A deficiency or whether the deposition of glycogen occurs in all epithelial metaplasia.

Summary. In the epithelial metaplasia of the rat uterus that occurred following vit. A deficiency, glycogen was observed in the stratified epithelium. An earlier report showed that the normal uterine epithelium of the rat uterus contains no glycogen.

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Effect of Methotrexate Therapy upon Choriocarcinoma and Chorioadenoma. (22757)

MIN CHIU LI, ROY HERTZ, AND DONALD B. SPENCER

National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Folic acid is known to be essential for the growth of the female genital tract and for normal embryonic development. Hertz *et al.* demonstrated the inhibition of estrogen-induced growth in the chick oviduct and monkey uterus in animals maintained on a folic acid deficient diet or treated with antagonists of either folic acid or adenine(1,2,3). Reduction of a previously high titre of gonadotropin occurred following a short course of methotrexate in a hypophysectomized woman with metastatic melanoma.*

The present report includes observations on the effect of a repeatedly administered and intensive regimen of methotrexate upon the urinary excretion of chorionic gonadotrophin and upon the clinical course in 2 patients with choriocarcinoma and one patient with chorioadenoma destruens.

Methods. The diagnosis was established by histological evidence. The initial extent of the disease was determined by physical examination and by radiological survey. Other observations included recording on each day of (a) fluid intake and output, (b) caloric intake, (c) the hemogram, and (d) the body weight. Each week x-rays were taken and measurement of palpable or visible lesions was recorded. Urinary gonadotropin determinations were carried out by a modification of the method of Klinefelter *et al.*(4). Gonadotropin values are expressed as "mouse uterine units" per 24 hours. The titres on

* Unpublished.

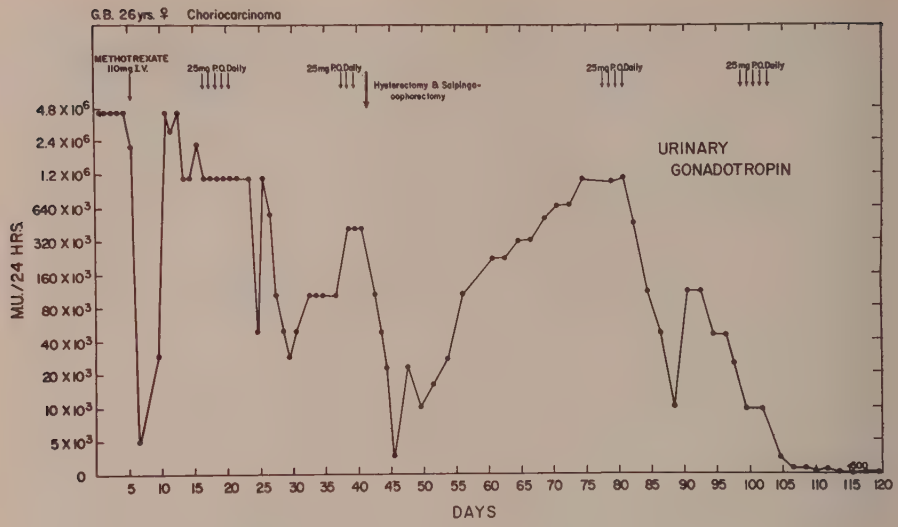


FIG. 1. Effect of intensive methotrexate therapy on urinary gonadotropin: Case 1.

graphs represent highest dilution yielding a positive reaction and twice this dilution was usually found to be negative. *Therapeutic Regimen*: The dosage regimen† of methotrexate is shown in the accompanying charts (Fig. 1, 2 and 3.) Two weeks after an initial single intravenous dose of 1 to 4.5 mg/kg body weight, there is given repeatedly at about 2 week intervals additional courses of 2.5 mg/kg body weight divided in 5 consecutive daily doses. The final course was given after the urinary gonadotropin titre had fallen to levels approaching normal with a view to a more sustained effect. Daily appraisal of the patient's hemogram, renal function, and clinical status with respect to stomatitis, glossitis, diarrhea, proctitis, and petechiae preceded the administration of the next dose. New courses were started only after clear evidence of complete hematological and gastrointestinal recovery from previous toxic effects.

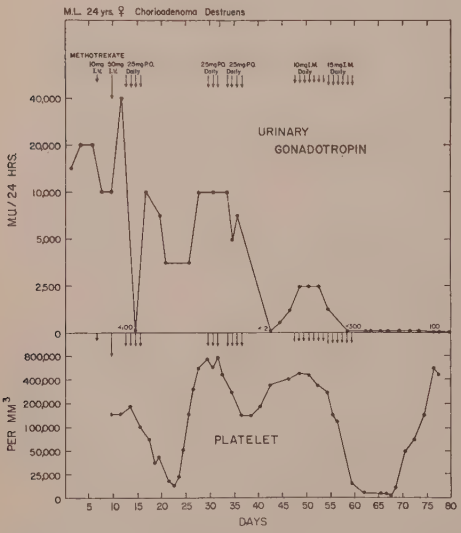


FIG. 2. Same as Fig. 1, and circulating platelet count: Case 3.

Results. By close adherence to the above mentioned precautions no undue toxicity was noted in these 3 patients. Experience with this same regimen (Fig. 3) emphasizes the possibility of renal impairment and this should be carefully checked by frequent BUN determinations and urinalyses. Moreover, preexisting renal impairment would constitute a definite contra-indication to the use of this regimen especially since any renal impairment would delay excretion and thereby enhance toxicity. Hepatic injury has not been observed.

† This regimen represents modification of procedure for intensive methotrexate administration devised by Dr. Paul T. Condit, Clinical Pharmacol. and Exp. Therap. Section, National Cancer Institute.

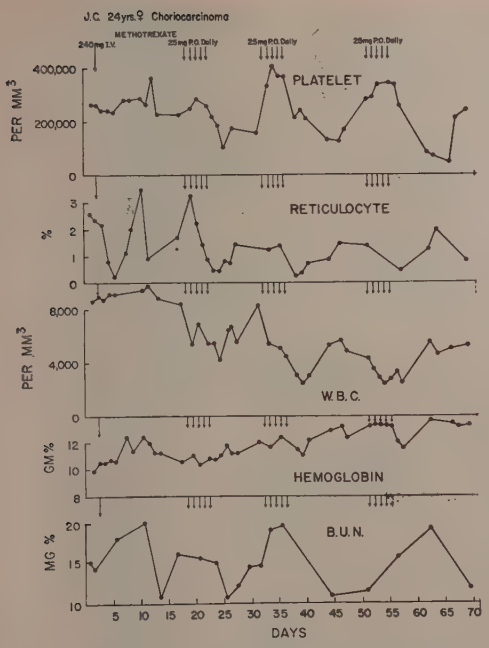


FIG. 3. Effect of intensive methotrexate therapy on the homopoietic elements and renal function: Case 2.

Fig. 2 and 3 illustrate transient impairment of hemopoiesis with each course of methotrexate. These effects began toward the close of each course and became most marked on the 4th to 10th day but full recovery was usually noted within 14 days.

Gastrointestinal difficulties such as anorexia, nausea, diarrhea, stomatitis, glossitis, ulceration of oral and rectal mucosa usually appeared and receded at about the same time as the bone marrow effects. These evidences of toxicity appeared to become progressively more apparent with each course of therapy.

Case 1. G.B., 26-year-old white housewife. She had a large pelvic mass and multiple bilateral pulmonary metastases. Diagnosis of choriocarcinoma had been established on tissue obtained by curettage. This case had been accepted for registration as choriocarcinoma in the Mathieu collection. On admission the patient had marked anorexia, protracted vomiting, and repeated bouts of uterine hemorrhage. There was evidence of marked weight loss and general debility. The urinary gonadotropin assay was positive at 4,000,000 mouse units/24 hours. The 5 courses of methotrexate given are indicated in Fig. 1. During the third course of methotrexate uterine bleeding became sufficiently profuse to indicate an emergency hysterectomy and salpingo-oophorectomy. Recovery was uneventful and further methotrexate therapy became indicated when the urinary gonadotropin titre had returned in a 6 weeks period from an immediate post-operative titre of 2,500 mouse units to 1,000,000 mouse units. After each course of methotrexate the gonadotropin titre was markedly suppressed (Fig. 1). With each successive course both the degree and duration of the gonadotropin reduction became greater. Following the 5th course the titre had receded to below 500 mouse units and has now remained at this reduced level for 5 weeks.

Correlated with this recession in urinary gonadotropin titre there was noted unequivocal x-ray evidence of regression of the pulmonary lesions (Fig. 4) and distinct amelioration of the patient's general clinical status.

Case 2. J.C., 24-year-old white housewife. She had a large, fixed, and irregular pelvic mass extending to the level of the umbilicus. She also had a globoid mass measuring 3 x 4 cm arising in the left labium major. Chest x-ray revealed diffuse bilateral pulmonary metastases. There had been extended vaginal hemorrhage and the patient was severely debilitated and critically ill. Several biopsy specimens showed choriocarcinoma with large masses of neoplastic cells presumably of trophoblastic origin. No villi were noted. The urinary gonadotropin titre was 200,000 mouse units/24 hours. Patient received 4 successive courses of methotrexate therapy. After the 4th course of therapy the gonadotropin titre had receded to below 200 mouse units and has remained at this level for the ensuing 6 weeks.

Accompanying this recession in gonadotropin titre the pelvic mass decreased to half its initial size and the labial mass was completely resolved. X-rays revealed striking clearing of the pulmonary lesions. The patient was completely rehabilitated and re-

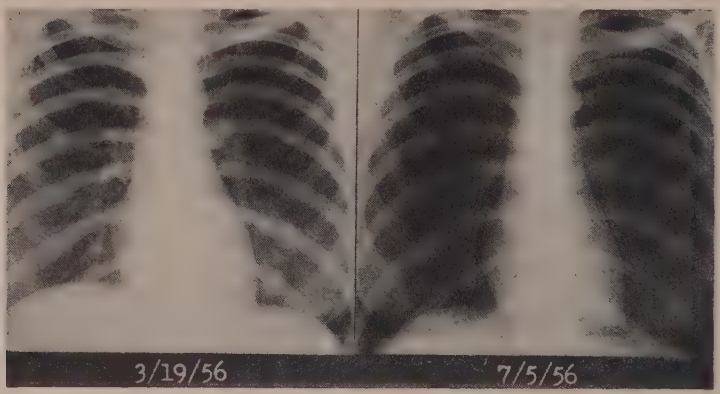


FIG. 4. Regression of pulmonary metastases. Case 1. Methotrexate therapy initiated 3/12/56.

mains under observation at this time on an ambulatory basis.

Case 3. M.L., 24-year-old housewife. Six months prior to admission she had undergone hysterectomy and bilateral salpingo-oophorectomy. The confirmed histological diagnosis was chorioadenoma destruens. On admission she was critically ill due to rapid progression of radiologically demonstrable pulmonary metastases. The urinary gonadotropin titre was 20,000 mouse units/24 hours.

Fig. 2 presents the therapeutic regimen and the associated changes in gonadotropin excretion in this case. It will be noted that the titre had dropped to below 300 mouse units and the patient experienced complete clinical recovery. During the past 8 months her gonadotropin titre has been found at monthly intervals to range between 100 and 200 mouse units. This titre is normal in our laboratory for an ovariectomized patient. Monthly x-rays (Fig. 5) show no evidence of metastatic disease since March this year.

Discussion. The correlation between clinical course and histopathological findings is notoriously confused in the case of trophoblastic tumors. Novak(5) and numerous others have described the pitfalls attendant upon clinical prognostication in this group of diseases on the basis of even the most expert histopathological analysis. Accordingly, we do not propose to draw any definitive therapeutic conclusions on the basis of the limited observations reported here. Nevertheless, the association of the as yet benign clinical course of these 3 patients with the methotrexate-induced recessions of urinary gonadotropin titre and regression of pulmonary metastases indicate the desirability of more extended study of the therapeutic potentialities of intensive treatment with antifolic and related compounds in this group of diseases.

Thiersch and Phillips(6) observed that the placental tissue in aminopterin-treated rats were not especially affected. This brings up the question whether methotrexate therapy employed here may have more general clinical application than in the type of cases just described. However, previous clinical experience with less intensive anti-folic regimens in several types of neoplasms other than acute leukaemia has not been encouraging(7). In any event, these observations emphasize the experimental advantage of an available index of tumor activity such as gonadotropin excretion in evaluation of therapeutic agents.

The intensive regimen of methotrexate therapy with courses interposed between successive periods of recovery from gastrointestinal and hematological toxicity is predicated upon the expected reversibility of such effects in normal tissues and possible failure of such reversibility in tumor tissue after repeated courses. The behavior of the urinary gonadotropin titres in these cases suggests that this expectation may have been realized. More prolonged follow-up in more cases will be required for further evaluation of such a rationale.

Caution was taken to ascertain whether the

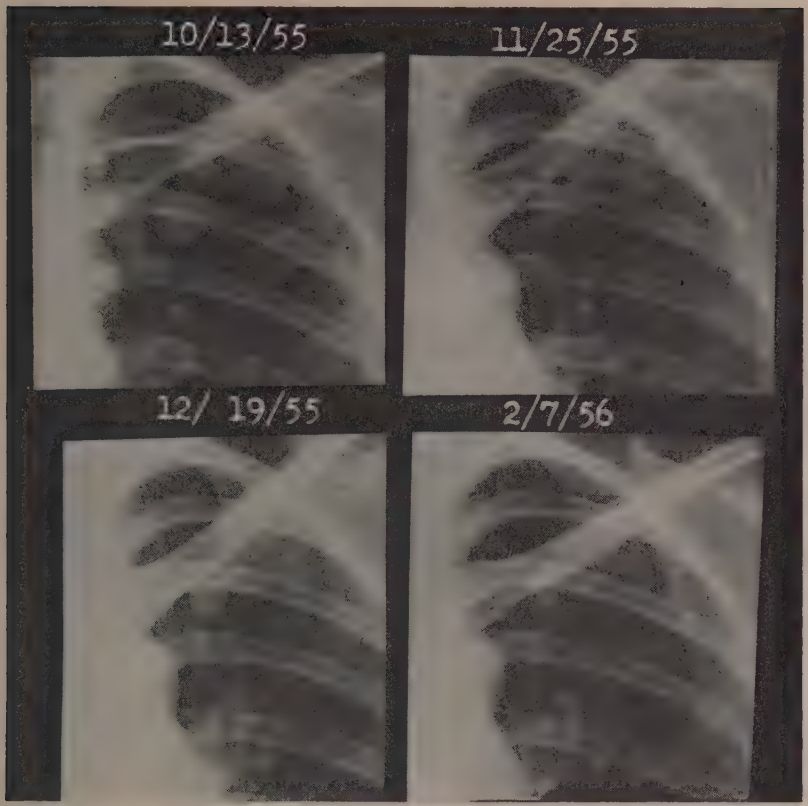


FIG. 5. Regression of pulmonary metastases. Case 3. Methotrexate therapy initiated 10/20/55.

apparent reduction in urinary gonadotropin may not have resulted from interference of excreted methotrexate and its metabolites with the biological assay. Addition of methotrexate to high titre urines did not alter the titre obtained probably because the dialysis of the urinary concentrate effectively removed substances of small molecular weight. Moreover, the persistence of the reduced titres for prolonged periods following cessation of therapy would render the possibility of such chemical interference unlikely.

Summary. 1) Two patients with choriocarcinoma and one with chorioadenoma destruens with proven progressive metastases were treated with methotrexate, a folic acid antagonist, according to intensive regimen of repeated courses of dosages producing substantial but reversible toxicity. Initially high urinary gonadotropin present in all 3 cases approached normal levels following this regimen and such low levels were sustained following cessation of treatment. 2) Unequivocal clinical improvement and radiological evidence of regression of pulmonary metastases was observed in all 3 cases. Nevertheless, extreme variations in pattern of clinical behavior of this type of tumor necessitate more observations before any definitive therapeutic implications may be drawn from these studies.

We are deeply grateful to Dr. Delbert M. Bergenstal, to Dr. Gordon Zubrod, and to Dr. Herbert A. Lubs for their valuable criticisms.

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Anti-Inflammatory Protection of Guinea Pigs by Egg Yolk: Effect on Adrenals and Serum Constituents. (22758)

IRENE CHANG AND CYRUS E. FRENCH* (Introduced by Hutton D. Slade)
Rheumatic Fever Research Institute, Chicago, Ill.

Coburn *et al.*(3) have shown that dietary egg yolk reduces the intensity of the inflammatory response induced by passive anaphylactic arthritis in young guinea pigs. Their observations indicated that the active material of egg yolk resides in the alcohol soluble (crude phosphatide) fraction and is absent from both the acetone solubles (neutral fats and cholesterol) and the yolk protein. We have confirmed the above findings(19) using the anti-inflammatory bio-assay method of French *et al.*(8). The adrenal hormone, cortisone, and its related steroids have been used in treatment of inflammatory diseases(15,18). Their protection of laboratory animals against inflammation induced by BCG(21), tuberculin(2), turpentine(16), and Pneumococci(22) has also been demonstrated. French *et al.* (8) showed that cortisone treatment also reduced swelling in joints of guinea pigs which had been subjected to a reverse Arthus reaction.

The present investigation was undertaken (a) to compare the anti-inflammatory protection by cortisone to that afforded by dietary egg yolk, (b) to study the effect of yolk alcohol solubles on adrenals of the guinea pig, and (c) the effect of egg yolk on blood constituents of guinea pigs.

Methods. 1. *Fractionation of egg yolk powder.*† Acetone solubles, alcohol solubles and the protein fraction of egg yolk were obtained by procedures of Coburn *et al.*(3). 2. *Assaying procedure.* Weanling guinea pigs weighing about 160 g (unless otherwise indicated) were used as test animals in groups of 6 or more. Each animal was weighed just prior to, and every 3 days during the experiment, and daily food intake was recorded. Egg yolk and yolk fractions were individually fed to guinea pigs as supplements to a basal control diet for a period of 21 to 26 days. The basal diet contained 50% Rockland guinea pig diet and 50% dixie rabbit pellets (22% protein) to which was added 0.1% ascorbic acid. At end of the feeding period, the guinea pigs usually reached the desired weight of approximately 300 g and then used for bio-assay(8). For each assay group, a control group of 6 or more guinea pigs was fed and challenged at the same time to minimize environmental influences on inflammation.

Results. Exp. 1 was a comparison of the anti-inflammatory activity of egg yolk and yolk alcohol solubles to that of cortisone. Cortisone acetate was injected intraperitoneally into 300 g guinea pigs (after 3-4 days on basal diet) 1 hr prior to assay. For each assay a second group of guinea pigs, pretreated with saline, was used as control. Feeding dried egg yolk at 19.5%, or yolk alcohol solubles at 2-8% of diet, resulted in 28-40% inhibition of swelling (Table I) similar to that obtained by administering cortisone acetate at the level of 25 mg/kg body weight(8). When alcohol solubles were fed at less than 2% of diet, no protection was observed.

Exp. 2 was a study of the effect of dietary yolk alcohol solubles on adrenals of the guinea

* Present address: Pennsylvania State University, University Park, Pa.

† Prepared by Wilson Laboratories, Chicago, Ill.

TABLE I. Effect of Feeding Dried Egg Yolk Powder and Yolk Alcohol Solubles on Mean Swelling Index (M.S.I.) in Reverse Arthus Reaction of Guinea Pigs.

No. guinea pigs	Fed control, M.S.I. ± S.E.	No. guinea pigs	Yolk powder, 19.5%	Yolk alcohol solubles 1%	2%	4%	6%	8%	% inhibition
				M.S.I. ± S.E.					
6	4.8 ± .37	8	3.4 ± .37						28
7	4.8 ± .34	6		5.6 ± .43					0
6	6.0 ± .30	5			4.3 ± .59				28
6	4.0 ± .37	7				3.0 ± .53			25
6	6.0 ± .30	6					3.9 ± .77		35
7	4.6 ± .41	6						3.4 ± .18	26
6	4.8 ± .31	6						2.9 ± .20	40
6	5.2 ± .50	7						3.1 ± .50	40

pig. Guinea pigs weighing 160 or 200 g were fed for periods of 21 and 10 days respectively. At completion of the assay, the guinea pigs were killed by blow on head. The adrenals were quickly removed, cleaned from adherent tissues, washed with physiological saline, weighed and frozen. They were kept in frozen state until needed. The adrenals in each group were pooled and water content was determined(1). The total lipid content was determined on ground, dried adrenals by extraction with hot chloroform and methanol (2:1) in Soxhlet extractor for 36 hrs. The extract was evaporated to dryness in water bath at 40°C under stream of nitrogen. The residue was redissolved in chloroform at room temperature and filtered. The total lipid was calculated from the weight of residue remaining after filtrate was dried to a constant weight. The total cholesterol in lipid extract was then determined(23).

The similarity between the anti-inflammatory protection afforded the guinea pig by dietary egg yolk and by injected cortisone acetate suggested that the egg yolk effect might be mediated by the adrenals. However, no indication of increased adrenal hormone production was observed in the treated guinea pigs (Table II). Adrenal weights were not increased nor were adrenal cholesterol levels depressed by feeding yolk alcohol solubles.

Exp. 3. The effect of dietary egg yolk on certain blood constituents of guinea pigs was studied. Serum lipids of guinea pigs, fed egg yolk, or 1 of 3 egg yolk fractions (alcohol solubles, acetone solubles and protein fraction) were determined. Each experimental diet contained one or another of the 3 fractions in a quantity equivalent to that which would be found if 19.5% of diet weight were egg yolk. In studying the effect of egg yolk on serum total N and non-protein N levels, the guinea pigs were fed the alcohol soluble fraction. Heart blood samples were obtained from fasting animals 24 hrs after completion of the assay. Total lipid was determined by extracting lyophilized serum with hot chloroform and methanol (2:1) as described for determination of adrenal lipid content. Lipid phosphorus and serum cholesterol were then determined(11,23). Phospholipid values were calculated by multiplying lipid phosphorus by 26. The serum total N and non-protein N of the protein-free filtrate(7) were determined by micro-Kjeldahl(13,14). No essential dif-

TABLE II. Effect of Yolk Alcohol Solubles on Adrenals of 6 Guinea Pigs in Each Series.

Treatment	Days	Wt, mg/100 g body wt	Water, %	Lipid, %*	Cholesterol, %†
Control	21	46.5 ± 4.5	57.1	50.5	25.3
Yolk alcohol solubles, 8%		38.3 ± 2.8	56.6	48.2	25.6
Control	10	37.1 ± 4.1	65.6	54.6	17.2
Yolk alcohol solubles, 2%		42.5 ± 3.5	63.7	47.2	20.7
" " " , 6%		42.3 ± 2.8	63.6	49.6	20.1

* Expressed as % of dry wt of adrenals.
† " " " " " lipid content of adrenals.

TABLE III. Serum Total Lipid, Phospholipid and Cholesterol Levels of Guinea Pigs on Control and Supplemented Diets.

Treatment	Total lipid, mg/ml	P	Phospholipid, mg/ml	P	Total cholesterol, mg/ml	P	C/P
Control	2.03 ± .12*		.384 ± .04*		.466 ± .07*		1.21
Dried yolk powder	4.79 ± .92	.0125	.483 ± .08	>.1	.606 ± .08	>.1	1.25
Control	2.14 ± .22		.324 ± .06		.258 ± .07		.80
Yolk protein	14.60 ± 1.17	.0025	3.774 ± .42	<.0025	7.410 ± .34	<.0025	1.96
Control	2.07 ± .22		.376 ± .04		.437 ± .05		1.16
Yolk alcohol solubles	3.55 ± .71	.025	.533 ± .05	.05	.468 ± .03	>.1	0.91
Control	2.78 ± .09		.406 ± .04		.354 ± .02		0.87
Yolk acetone solubles	4.42 ± .80	.025	1.074 ± .29	.025	1.046 ± .31	<.025	0.97

* ± S.E.

ference was found in average daily food intake and weight gain between guinea pigs fed control or supplemented diets. Animals, which were fed yolk alcohol solubles, received the same protection as those fed a supplement of dried yolk powder. Both the protein and the acetone soluble fractions appeared inactive.

Guinea pigs which had received acetone soluble material possessed enlarged fatty livers and gallbladders. Guinea pigs from other groups had normal organs.

Feeding dried egg yolk and yolk alcohol-solubles resulted in elevation of serum total lipid with no increase in cholesterol to phospholipid ratio (Table III). Feeding inactive yolk fractions caused elevation of serum total lipids, phospholipids, cholesterol, and the cholesterol to phospholipid ratio. The serum total N and non-protein N were unaffected by feeding yolk alcohol solubles.

Discussion. An anti-inflammatory effect of phospholipid, sphingomyelin has been demonstrated by several workers. Kerr *et al.*(12) showed that sphingosine, as well as sphingomyelin, inhibited the skin reaction in cattle sensitized to *Trichomonas foetus* antigen. The favorable effect of sphingomyelin and sphingosine has also been observed in guinea pigs sensitized to tuberculin(6).

In preliminary fractionation of alcohol solubles of egg yolk, we found that the active factor was present in the remaining fraction of alcohol solubles, after lecithin had been precipitated. The lecithin fraction which was obtained by a cadmium chloride precipitation did not appear to be active. The possibility that the active yolk material is also associated with the crude sphingomyelin fraction has been suggested by Coburn *et al.*(3).

Of the serum constituents studied, the total lipid fraction was elevated by feeding egg yolk or yolk alcohol solubles. Serum phospholipids and cholesterol were slightly elevated by these two diet supplements. Therefore, it seems that elevation of certain serum lipids other than cholesterol and phospholipids accounts for the hyperlipemia observed after feeding active egg yolk fractions. Hyperlipemia produced by active yolk material is quite comparable to that observed in rat, rabbit and human after administration of cortisone(5,4,10,20). These reports indicated that administration of cortisone caused hyperlipemia due chiefly to increase of lipids other than cholesterol and phospholipids. The inactive yolk fractions both caused an increase in serum total lipids proportional to the increase of phospholipids and cholesterol. If the inhibitory effect of the active factor on the experimental inflammation in guinea pigs depends on changes in the blood lipid fractions, then it seems logical to attribute its effect to the associated increase in lipid fractions other than cholesterol and phospholipids. However, the possibility that active yolk material inhibits induced inflammation independent of its effect on blood lipids cannot be excluded at present.

Summary. The anti-inflammatory activity of egg yolk has been studied in weanling

guinea pigs. 1. The active material resided in the alcohol soluble fraction and was absent from the protein and acetone soluble fractions. 2. The anti-inflammatory effect did not appear to be mediated by the adrenals. 3. Feeding egg yolk and yolk alcohol solubles resulted in an elevation of serum total lipids with no increase in cholesterol to phospholipid ratio. Inactive yolk fractions caused elevations of serum total lipids, phospholipids, cholesterol, and cholesterol to phospholipid ratio. 4. Serum levels of total N and non-protein N were unaffected by yolk alcohol solubles.

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Δ^1-Hydrocortisone and Hydrocortisone: Plasma 17-Hydroxycorticosteroid Concentrations in the Dog Following Oral and Intravenous Administration. (22759)

Elliott J. Collins, Arlington A. Forist, and Esther B. Nadolski
Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Δ^1-Hydrocortisone ($\Delta^{1,4}$-pregnadiene-11β, 17α, 21-triol-3,20-dione) has received considerable attention as a potent synthetic analogue of hydrocortisone (Δ^4-pregnene-11β, 17α, 21-triol-3, 20-dione). Clinical studies have indicated Δ^1-hydrocortisone to be 3 to 5 times as potent as the parent steroid(1-3). Evaluation of glucocorticoid activity (liver glycogen deposition, eosinopenic response, thymus involution) has shown a similar ratio (4). Introduction of unsaturation at the C_1 position of the steroid A-ring could conceivably alter the rates of absorption and/or metabolism of the compound. In the present study, this possibility has been examined experimentally in the dog by determining the changes in plasma 17-hydroxycorticosteroid (17-OHCS) concentrations as a function of time following oral and intravenous administration of Δ^1-hydrocortisone and hydrocortisone. Subsequent to this study, a similar evaluation of Δ^1-hydrocortisone in the human has been reported(5).

Materials and methods. Male mongrel

dogs weighing 15-20 kg were used in these studies. The animals were fasted for 16-18 hours prior to the steroid administration and food but not water was withheld during the blood sampling period. In experiments involving oral administration, the preparations tested were as follows: (a) Δ¹-hydrocortisone: 5 mg (5 expts), 20 mg (4 expts), and 40 mg (4 expts); (b) hydrocortisone: 10 mg (4 expts), 20 mg (23 expts), and 40 mg (4 expts). Following administration of the compressed tablets, 3-6 blood samples (25-45 ml) were collected over periods of 2-5 hours from the external jugular vein without anesthesia. For the intravenous studies, the steroids were administered as the free alcohols (1 mg/ml in 10% ethanol-isotonic saline) and as the 21-hemisuccinate sodium salts (equivalent to 1 mg of free steroid/ml in isotonic saline) by a rapid infusion into the antecubital vein at a dose of 2 mg/kg. During a 3-4 hour period, 7-8 blood samples were collected. Plasma 17-OHCS concentrations were determined by a modification of the Nelson-Samuels procedure(6,7,8). In order to avoid frequent occurrence of cloudy solutions in the spectrophotometric step, it was necessary to partition the residue from the chloroform extract of dog plasma between 70% ethanol and Skellysolve B. The 70% ethanol extractables were then carried through Florisil chromatography as described by Nelson and Samuels(6). The spectrophotometric procedure was modified to include a direct correction for non-specific, acid-produced chromogens(8) similar to that originally recommended by Porter and Silber (9).

Results. Before applying routine 17-OHCS procedures in studies involving Δ¹-hydrocortisone, it was necessary to know the reactivity of this steroid with the phenylhydrazine-sulfuric acid reagent as well as its behavior on Florisil columns. The absorption spectra of the phenylhydrazine-sulfuric acid chromogens obtained from Δ¹-hydrocortisone and hydrocortisone are shown in Fig. 1. Absorptivity at the maximum is less for Δ¹-hydrocortisone than for hydrocortisone and there is a slight shift of the maximum toward longer wavelengths due to the effect of the unsaturation

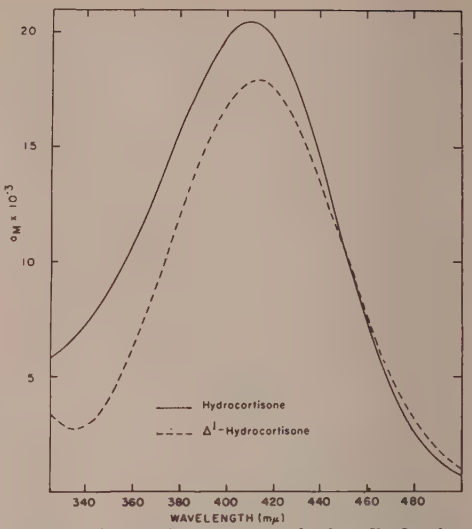


FIG. 1. Absorption spectra of phenylhydrazine-sulfuric acid chromogens from Δ¹-hydrocortisone and hydrocortisone (a_M calculated on basis of steroid conc. in reaction mixture).

at C_1. Routine measurements for both steroids were made at 410 mμ. By the procedure employed(8), the calibration curves at this wave length obey Beer's law over a wide range, and Δ¹-hydrocortisone is 87% as chromogenic as hydrocortisone. Recovery of Δ¹-hydrocortisone from Florisil columns was slightly but consistently less than hydrocortisone. With a typical batch of Florisil, recoveries (mean ± s.e.m.) were as follows: Δ¹-hydrocortisone, 88.1 ± 1.1% (35 samples); hydrocortisone, 90.4 ± 1.4% (14 samples). For these reasons, the steroid administered was used as reference standard for chromatography and spectrophotometry.

To interpret data following intravenous administration of the steroid hemisuccinates, the behavior of hydrocortisone-21-hemisuccinate during Florisil chromatography was examined. None of the ester was eluted by either 2% or 25% methanol-in-chloroform. Further elution with 40% methanol-in-chloroform recovered about 30% of the ester. Therefore, even though some of the ester was extracted from plasma, it was removed during chromatography and only free 17-OHCS were measured. Previous studies have shown that neutral esters such as the acetate and cyclopentylpro-

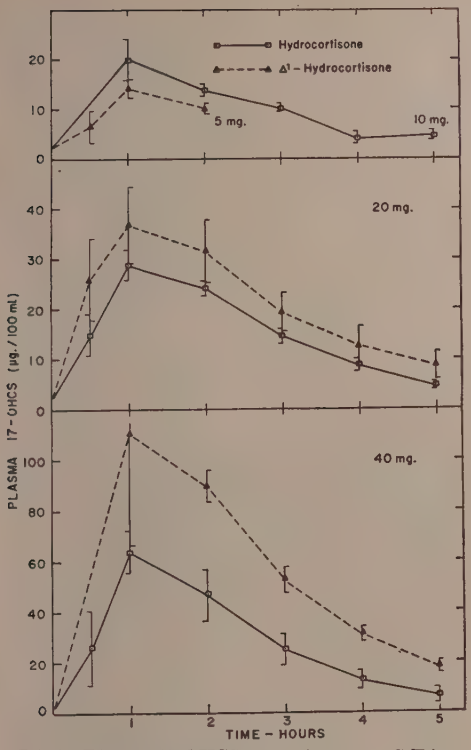


FIG. 2. Plasma 17-OHCS conc. (mean ± S.E.) vs. time following oral admin. of Δ^1-hydrocortisone and hydrocortisone.

pionate also are not eluted from Florisil in the 17-OHCS-containing 25% methanol-in-chloroform fraction(8).

Mean plasma 17-OHCS concentrations as a function of time following the oral administration of Δ^1-hydrocortisone and hydrocortisone are shown in Fig. 2. At a dose of 5 mg, Δ^1-hydrocortisone produced elevations in plasma 17-OHCS concentrations of about 70% of those obtained with 10 mg of hydrocortisone. At the 20 mg level, Δ^1-hydrocortisone gave plasma 17-OHCS levels which were higher at the maximum and which remained higher throughout the experimental period. At the 40 mg dose, the higher maximum values and prolonged elevations were even more striking. In addition, although 20 mg of Δ^1-hydrocortisone produced maximum plasma 17-OHCS concentrations of only about 60% of those observed after 40 mg of hydrocortisone, the response curves crossed after about 4 hours. This indicated a difference in the rates of removal of the 2 steroids from the circulation.

Plasma 17-OHCS half-life values obtained following the intravenous administration of Δ^1-hydrocortisone and hydrocortisone as the free steroids and as the sodium hemisuccinates are shown in Table I. Typical first order regression curves (method of least squares) are shown in Fig. 3. The mean half-lives of Δ^1-hydrocortisone and hydrocortisone were 71.3 and 52.1 minutes respectively. When administered as the sodium hemisuccinates, the mean plasma 17-OHCS half-lives were 79.7 and 67.0 minutes respectively. Therefore, Δ^1-hydrocortisone is removed from the circulation more slowly than hydrocortisone in the dog.

Discussion. The data presented indicate that orally administered Δ^1-hydrocortisone produced greater and more prolonged elevations of plasma 17-OHCS in the dog than did equivalent doses of hydrocortisone. The half-life of intravenously administered Δ^1-hydrocortisone was also longer than that of hydrocortisone (ratio 1.37). Further, a comparison of the ratios of plasma 17-OHCS concentrations one hour after oral administration shows the following: 10 mg (assuming linearity of

TABLE I. Half-Lives of Δ^1-Hydrocortisone and Hydrocortisone in the Dog.

	Half-life (min. ± stand. dev.)			
	Δ^1-Hydrocortisone		Hydrocortisone	
Dog	Alcohol	Hemisuccinate	Alcohol	Hemisuccinate
500	69.1 ± 2.1	89.8 ± 6.4	51.0 ± 2.2	59.7 ± 5.4
503	70.4 ± 2.0	—	45.1 ± .9	—
505	76.2 ± 3.9	72.8 ± 3.0	59.2 ± 1.4	87.3 ± 2.8
508	69.3 ± 1.6	—	53.1 ± 2.0	57.7 ± 2.2
502	—	76.4 ± 3.1	—	63.1 ± 2.2
Mean ± S.E.	71.3 ± 1.7	79.7 ± 5.2	52.1 ± 2.9	67.0 ± 6.9

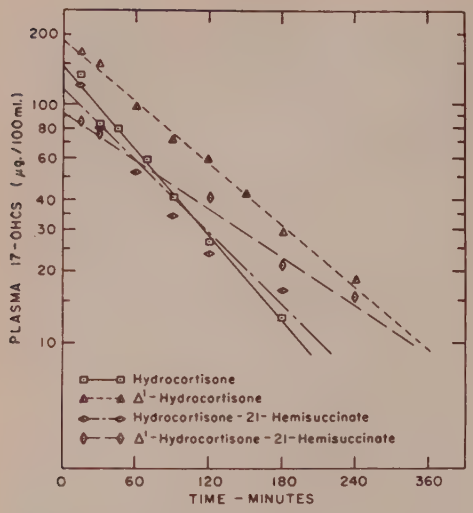


FIG. 3. Typical first order regression curves following intrav. admin. of Δ¹-hydrocortisone, hydrocortisone and the respective hemisuccinates.

response between 5 and 10 mg), 1.42; 20 mg, 1.28; and 40 mg, 1.73. Similarity of these ratios to the ratio of half-lives suggests that there is no appreciable difference in the rates of absorption of Δ¹-hydrocortisone and hydrocortisone from the gastrointestinal tract of the dog. It therefore appears that the higher and more prolonged elevations of plasma 17-OHCS following oral administration are a reflection of the reduced rate of metabolism of the Δ¹-steroid.

With the intravenously administered steroid hemisuccinates, the plasma 17-OHCS half-lives were greater than observed with the corresponding free alcohols (Table I). These values are not half-lives of the administered esters since only free 17-OHCS are measured by the analytical procedure. Apparently, hydrolysis of the hemisuccinates proceeds at a rapid but finite rate and the procedure determines the intermediate in a series of consecutive reactions such as: steroid hemisuccinate → 17-OHCS→ metabolite(s). Jakoby and Tomkins(10) have studied the metabolism of pregnan-21-ol-3,20-dione-21-hemisuccinate by rat liver preparations and have reported that hydrolysis of the ester, followed by reduction of the C_3 carbonyl, analogous to the scheme above, represents the main metabolic pathway. However, they also observed considerable reduction of the C_3 carbonyl without prior hydrolysis. The Δ¹-hydrocortisone and hydrocortisone hemisuccinates probably behaved similarly. The somewhat larger standard deviations obtained (Table I) when the data are fitted as a first order regression (Fig. 3) indicate that the plasma 17-OHCS half-lives so obtained represent the simplification of a complex series of consecutive reactions. It may only be concluded that intravenous administration of the steroid esters results in somewhat lower but, due to continuing hydrolysis of the esters, more prolonged elevations of plasma 17-OHCS.

Metabolism of corticosteroids containing the Δ⁴-3-ketone structure proceeds via reduction of first the C_4 unsaturation and then the C_3 carbonyl to produce tetrahydro derivatives which are subsequently conjugated(11). Alternatively, some reduction of the C_{20} carbonyl may also occur(12,13,14). The longer half-life of Δ¹-hydrocortisone may reflect the rate of an additional reduction at C_1 prior to the normal metabolism or may reflect a change in the relative proportions of the alternative processes. The recent studies by Liddle and Richard(15) and by Silber and Morgan(16) have shown that alteration of ring A or substitution of fluorine at C_9 of the hydrocortisone molecule results in a reduced rate of metabolism. These investigators have suggested, on the basis of urinary excretion studies, that changes in the steroid molecule alter its susceptibility to the usual enzymatic attack on ring A. Glenn* has observed that the 2-methyl-, 9α-fluoro-, and Δ¹-derivatives of hydrocortisone are metabolized at a markedly reduced rate, when compared with hydrocortisone, in a microsome-supernatant fraction of rat liver. Hence, it appears that enhanced potencies of these steroids are related to a reduced rate of metabolism in the animal. Final evaluation of the processes involved awaits further experimentation.

Summary. The effects of orally and intravenously administered Δ¹-hydrocortisone and hydrocortisone on plasma 17-OHCS concentrations in the dog have been studied. Δ¹-

* Unpublished data.

Hydrocortisone produced higher and more prolonged plasma 17-OHCS elevations than did equivalent doses of hydrocortisone administered orally. The half-life of Δ^1-hydrocortisone was found to be 1.37 times that of hydrocortisone. Intravenous administration of these steroids as the hemisuccinates gave lower but somewhat more prolonged elevations in plasma 17-OHCS concentrations than the corresponding free alcohols. It was concluded that Δ^1-hydrocortisone was metabolized more slowly than hydrocortisone and suggested that this may explain, at least in part, the enhanced potency of the Δ^1-steroid.

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Titration of Influenza Virus and Viral Antibodies by Preserved Human Erythrocytes.* (22760)

CHARLES D. COX AND E. C. PIRTLE

Department of Microbiology, University of S. Dakota School of Medicine, Vermillion

Procedures for titration of influenza viruses and their antibodies by use of chicken and human erythrocytes in tube hemagglutination (HA) and hemagglutination inhibition (HAI) tests have been well established(1-8). Most of the presently used tube HA and HAI titrations are read by the pattern method(5) and employ freshly washed chicken or human erythrocyte suspensions. Flick(9) reported the use of formalin treated human erythrocytes in studying the hemagglutinating capacity of influenza A virus; however, these cells exhibited spontaneous agglutination and could not be read by the pattern technic. Previous work in this laboratory(10) has demonstrated effective preservation of sheep erythrocytes for use in various serological systems. This report describes the preparation and use of preserved human erythrocytes in the titration of influenza viruses and their antibodies.

Materials and methods. Diluent used in all serological tests consisted of 0.137 M NaCl, 0.01 M Na_2HPO_4, and 0.003 M KH_2PO_4 in distilled water, the pH being 7.2 $\pm$ 0.05. Blood group O, Rh+ human erythrocytes were used in all tests. Those used as fresh cell suspensions were obtained by bleeding into an equal volume of Alsever's solution, and before use they were washed 3 times in 40 volumes of diluent and resuspended to a final concentration of 0.5%. Egg-adapted departmental strains of the type A (PR8 and IA43),

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type A' (Benson and USD 2/53), and type B (Lee) influenza viruses were used throughout this study, representing pools of chorioallantoic and amniotic fluid viruses respectively. IA43 strain was originally obtained from Dr. A. P. McKee, University of Iowa, and the Benson strain from Dr. Jonas Salk, University of Pittsburgh. Virus preparations were stored at –20°C. Antiviral sera were prepared as pools from several immunized rabbits and stored at –20°C. Normal rabbit serum was prepared in a similar fashion from non-immunized rabbits. Portions of pre-immunization sera, as well as immune sera, were treated with receptor-destroying enzymes (RDE) prepared from filtrates of the 4Z strain of *Vibrio cholerae* according to the method suggested by the Expert Committee on Influenza(8). Following treatment with RDE all sera were inactivated by heat at 56°C for one hour. *Preparation of Preserved Human Erythrocytes (PHE).* PHE were prepared in a manner similar to that previously reported for preparation of sheep erythrocytes(10), with two major exceptions. Defibrinated group O, Rh+ human blood was always used, and erythrocytes were exposed to formalin twice. Forty ml of 50% formalin-diluent (equal volumes of formalin and twice concentrated diluent above) were rapidly mixed with 100 ml defibrinated human blood previously diluted with an equal volume of diluent. Formalinized blood was placed in a 37°C waterbath for 1½ hours with frequent mixing. Cells were then centrifuged and washed twice in at least 50 volumes of diluent each time, taking care to remove and discard by aspiration the top thin light colored coat after the first centrifugation. The sediment was reconstituted to 100 ml in diluent, again rapidly mixed with 40 ml of 50% formalin diluent, and held in the 37°C waterbath for another 1½ hour interval. The cells were then centrifuged and washed 6 times in at least 50 volumes of diluent each time. The washed and packed erythrocytes were then resuspended in diluent containing 0.3% formalin to approximately 10% by volume. Cell volume was then more accurately adjusted to 4% by centrifuging 5.0 ml of the 10% suspension in a Kolmer or vaccine tube and determining the volume of packed cells. As an additional precaution against contamination during use of the cells after preparation, 0.25 ml of a dye mixture (1 part of 1% aqueous crystal violet plus 2 parts of 1% aqueous brilliant green) was added and mixed with each 100 ml of 4% suspension. Addition of the dye also facilitated reading. PHE was dispensed in screw-cap bottles or dropper-vials and stored in the refrigerator. Tube HA and HAI titrations were carried out with 0.5% suspensions of both freshly washed human erythrocytes and PHE as described by the Expert Committee on Influenza(8), with the exception that HAI titers are expressed as reciprocals of final dilutions of sera. *Rapid Plate Titration of Influenza Virus.* CAF virus, undiluted and diluted 10^{-1} and 10^{-2}, was pipetted onto appropriate 2 cm rings on a glass plate, each virus dilution in volumes of .08, .04, .02, and .01 ml from left to right. Virus was pipetted with Bangs pipettes or 0.2 ml pipettes graduated in 0.01 ml divisions, and 1 drop (.025 ml) of PHE was immediately added to each ring. All 4 volumes of each virus dilution were mixed with PHE with a separate applicator stick from right to left, rotated for 1 to 2 minutes by hand and read macroscopically against a dark background over a strong oblique light in the usual manner of reading rapid plate bacterial agglutination tests. Each virus was run simultaneously with a diluent or CAF control. Undiluted virus volumes of .08, .04, .02, and .01 ml were found to correspond to virus dilutions of 1:100, 1:200, 1:400, and 1:800 respectively in the tube HA procedure; volumes of virus diluted 10^{-1} corresponded to tube HA virus dilutions 10 times those with undiluted virus, etc. HA titer of the virus was expressed as the highest corresponding dilution showing definite clumping, with negative diluent or CAF controls showing fine suspensions of cells, smoothly dispersed. Thus, if .02 ml of virus diluted 10^{-1} was the smallest amount showing definite clumping, the titer would be 1:4000. *Rapid Plate Titration of Virus Antibody.* All viral antisera were titrated against dilutions of CAF virus calculated to contain 5 HA units per .02 ml dose. Thus, if the virus titer were 1:2000 (2000 HA/ml), 5 HA would be contained in .02 ml

undiluted virus; if virus titer were 1:8000, .02 ml virus diluted 1:4 would contain 5 HA. Inactivated serum, undiluted and diluted 10^{-1} and 10^{-2}, was pipetted onto appropriate rings on a glass plate, each serum dilution in volumes of .08, .04, .02, and .01 ml. Five HA units virus in .02 ml were then delivered to one side of each quantity of serum and 1 drop of PHE added. Reagents were mixed and read as indicated above. Undiluted serum volumes were found to correspond to tube HAI serum dilutions of 1:20, 1:40, 1:80, and 1:160 respectively, and decimal dilutions of serum corresponded to 10 fold tube HAI serum dilutions. Serum titer was expressed as highest corresponding dilution showing complete inhibition of agglutination, with diluent or negative serum controls showing marked agglutination.

Results. Preliminary observations on preparation of PHE paralleled those with preserved sheep erythrocytes(10). Two exposures to a final concentration of 14% formalin seemed optimal. Microscopically, in both stained and diluent preparations, the preserved erythrocytes stained well with some erythrocytes showing evidence of crenation. The addition of formalin diluent to whole blood rather than washed erythrocytes seemed advantageous; when washed erythrocytes were used, as reported by Flick(9), considerable lysis occurred. Furthermore, PHE showed considerable tendency to autoagglutinate when washed erythrocytes were exposed to final concentrations of formalin approaching 50%. PHE was found to be stable for several months, both from the standpoint of hemolysis and reactivity with influenza virus. Stable suspensions of PHE have been consistently prepared and maintained for a minimum of one year in the refrigerator. PHE suspensions were resistant to lysis in distilled water. A study of the optimal concentration of PHE for use in rapid plate procedures revealed that much less than a 4% suspension was too light to be easily read and too sensitive in the sense that the suspension seemed too granular. Much greater than 4% suspension was too heavy for easy reading and proved insufficiently sensitive with respect to the assigned virus dilutions when titrated by the rapid plate technic against known viruses. A 4% suspension was consistently found to yield end points in the rapid plate procedure in close agreement with tube hemagglutination titers, when virus dilutions were assigned to PHE-virus mixtures as described in above procedure for rapid plate titration of virus. PHE was used as 0.5% suspensions in all tube HA and HAI tests, in conformity with established procedures employing freshly washed human erythrocytes.

TABLE I. Comparison between PHE and Fresh Human Erythrocytes in Tube HA Titrations.

Virus	Virus pool	HA, units/ml	
		Fresh RBC	PHE
PR8 (A)	1	2560	1280
	2	6400	6400
	3	5620	7680
IA43 (A)	1	2560	5120
	2	9600	12800
	3	15260	30520
IA43 (A) inact.*	2	3200	6400
Benson (A′)	1	1920	2560
	2	3200	6400
USD 2/53 (A′)		300	400
Lee (B)	1	120	120
	2	1600	2400
	3	1920	1280

* Inactivated at 56°C for 1 hr.

It is apparent (Table I) that PHE compared favorably with suspensions of freshly washed human erythrocytes in titrating various influenza viruses. In like manner, PHE and fresh human erythrocytes showed comparable activity in HAI titrations of antiviral sera produced in rabbits (Table II). The rapid plate technic employing PHE showed good correlation with tube procedures in both HA and HAI titrations (Tables III, IV).

TABLE II. Comparison between PHE and Fresh Human Erythrocytes in Tube HAI Titrations.

Antivirus*	RBC	Virus		
		PR8	Benson	Lee
PR8 (A)	Fresh	10240†	1280	320
	PHE	20480	1280	320
Benson (A′)	Fresh	1280	5120	640
	PHE	640	5120	640
Lee (B)	Fresh	1280	640	10240
	PHE	640	1280	10240

* Antisera inactivated at 56°C for 30 min., but not RDE treated.

† Titers expressed as HAI units/ml.

TABLE III. Comparison between Tube and Rapid Plate HA Titrations Using PHE.

Virus	Virus pool	HA, units/ml Tube	Rapid
PR8 (A)	5	2560	2000
	6	6400	4000
	3	7680	8000
Lee (B)	5	2560	2000
	6	6400	4000
	3	1280	1000
IA43 (A)	3	30520	20000
Benson (A′)	3	2560	2000

Discussion. Data in Table I illustrate typical findings in many tube HA titrations using both PHE and fresh human erythrocytes, discrepancies between which rarely have exceeded 1 full tube dilution. Good correlation exists when titrating A, A′, and B types of influenza virus, and it is interesting to note that PHE is equally susceptible to agglutination by influenza virus inactivated by heat. PHE shows equally good correlation with freshly washed human erythrocytes in both sensitivity and specificity in reciprocal HAI titrations of antiviral sera untreated (Table II) and treated with RDE (Table IV). Additional experiments involving tube and rapid plate HA and HAI titrations with both PHE and freshly washed human erythrocytes on serially diluted virus and antiviral sera have shown equally good correlation. PHE offers the distinct advantages of employing constant cellular antigens and eliminating the daily washing of erythrocytes in viral and antiviral titrations. In our experience the presently described rapid plate procedure using PHE provides an extremely rapid and sufficiently accurate method for titrating influenza virus and viral antibodies.

TABLE IV. Comparison between Tube and Rapid Plate HAI Titrations Using PHE.

Antivirus*	Titration	Virus PR8	Virus Lee
PR8 (A)	Tube	10240†	< 40
	Rapid	4000	<100
Lee (B)	Tube	< 40	5120
	Rapid	<100	4000
Normal rabbit serum	Tube	< 40	< 40
	Rapid	<100	<100

* All sera RDE treated and inactivated at 56°C for 1 hr.

† Titers expressed as HAI units/ml.

Summary. The preparation of PHE and their use in both tube and rapid plate HA and HAI procedures for the titration of influenza virus and viral antibodies are described. Such preserved cells have been stable, with respect to both resistance to lysis and sensitivity to hemagglutination by influenza virus, for a year. PHE showed good correlation in sensitivity with freshly washed human erythrocytes, and the rapid plate technic using PHE correlated well with tube HA and HAI procedures.

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Radioactive Rose Bengal Test as a Quantitative Measure of Liver Function. (22761)

JEROLD M. LOWENSTEIN* (Introduced by A. L. Bloomfield)

Department of Medicine, Stanford University School of Medicine, San Francisco, Calif.

The radioactive rose bengal test, first described by Taplin, Meredith and Kade(1), promises to be one of the most sensitive known tests of liver function. By placing a radiation detector over the liver and injecting a small amount of I^{131}-tagged rose bengal intravenously, one can measure the rate of uptake and excretion of the dye. Normally, the activity rises to a peak in about 30 minutes and then falls as the dye is excreted via the biliary system into the bowel (Fig. 1). Where liver function is impaired, the rate of rise or fall, or both, is slower than normal. The original method for interpreting these uptake and excretion curves was semiquantitative, based on a visual comparison of each curve with a cluster of "normal" curves. Variations in size of patient, liver, or dose of tracer make this method quite unreliable. A purely quantitative analysis is described in the rest of this article.

Method. It is apparent that, at any time, liver radioactivity (L) is equal to the difference between the amount of activity taken up $(1-U)$ and the amount excreted $(1-E)$, symbolically, $L = (1-U)-(1-E)$. Therefore, $L = E-U$. Both U and E decrease logarithmically with time; therefore, each of these components is characterized by a constant, which we may call "uptake halftime" and "excretion halftime," by analogy with the "halflives" of radioactive elements. The uptake halftime is the period in which the liver takes up half the circulating dye from the blood; the excretion halftime is the period in which half the amount taken up is excreted through the bile ducts.

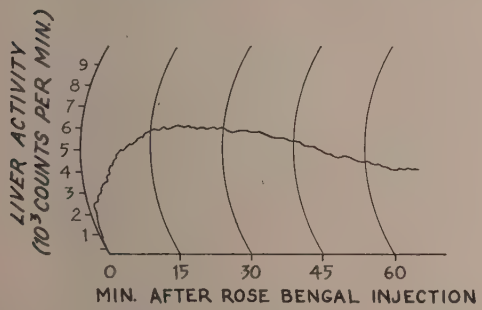


FIG. 1. A normal I^{131} rose bengal uptake excretion curve.

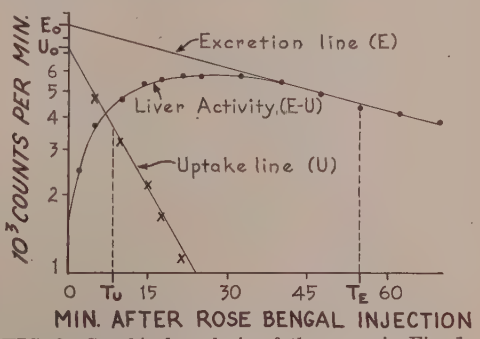


FIG. 2. Graphical analysis of the curve in Fig. 1. The uptake halftime (T_U) is 8 min., the excretion halftime (T_E) 55 min., and the liver blood volume $\left(\frac{E_o-U_o}{E_o}\right)$ is 18% of the total blood volume.

If we consider as "time zero" the time after radioactive rose bengal has been injected into the bloodstream but before any has been taken up by liver cells, then, at this time: Liver radioactivity = radioactivity of blood in liver $= E_o - U_o$ and since E_o represents the total radioactivity injected,

$$R \equiv \frac{\text{Liver Blood Volume}}{\text{Total Blood Volume}} = \frac{E_o-U_o}{E_o}.$$

The shape of each curve is determined by the 3 quantities, uptake halftime (T_U), excretion halftime (T_E), and fractional liver blood volume (R), and these constants can be found by graphical analysis.

Uptake excretion curves were obtained by the method of Taplin, Meredith and Kade(1) using a lead-shielded scintillation detector placed over the liver and aimed away from the gall bladder, with a counting rate meter

* Fellow of National Foundation for Infantile Paralysis 1955-56.

TABLE I. Summary of Mean Values of Quantitative Radioactive Rose Bengal Test.*

Clinical diagnosis	No. of patients	Uptake halftime, min.	Excretion halftime, min.	Liver blood vol, % of total blood vol
Normal	35	9	92	18
Acute hepatitis	3	11	185	32
Recovered hepatitis	7	9	129	20
Laennec's cirrhosis	18	18	300	32
Biliary cirrhosis	3	28	2800	35
Heart failure	2	21	106	24
Acute ulcerative colitis, with liver damage	1	7	260	23
Same case, after improvement of colitis		6	145	18
Common bile duct obstruction (carcinoma of head of pancreas)	1	15	2200	23
Same case, 1 wk after surgery (Whipple procedure)		9	140	21

* These values were derived from 42 uptake excretion curves published by Taplin, Meredith and Kade, and 28 curves recorded at Stanford Hospital and the Palo Alto Clinic. Comparison of the two groups reveals very nearly the same avg values for patients with the same diagnosis. The stand. dev. of each value is close to $\pm$ 25%.

and recorder. Each patient was given intravenously about 25 μg of rose bengal in 1 cc of saline, having activity of about 25 μc. Each record was continued until the peak activity was passed and the counting rate was well on the down slope (Fig. 1). Each curve is analyzed as follows. After background activity is subtracted, the curve is transposed to semilogarithmic paper (Fig. 2). A straight line (E) is drawn from the ordinate through the descending limb; this is the excretion line. The uptake line (U) is obtained by plotting differences between E and the rising portion of the curve, and drawing another straight line from the ordinate through these points. The uptake halftime is the time when $U = \frac{1}{2} U_o$, the excretion halftime is the time when $E = \frac{1}{2} E_o$. Fractional liver blood volume, $R = \frac{E_o - U_o}{E_o} \times 100\%$.

Results. Average values derived from data in this clinic and from the published curves of Taplin, Meredith and Kade(1) are presented in Table I. The standard deviation is about $\pm$ 25% in each group. The cases of ulcerative colitis and carcinoma of the head of the pancreas show how the test results in single individuals change with altered clinical state.

Discussion. From this series, it appears that hepatocellular damage is manifested by delayed uptake and excretion, obstructive processes cause delayed excretion, and circulatory failure, as expected, reduces the uptake rate. This test appears to have great practical value in the differential diagnosis of jaundice and liver disease.

Summary. The radioactive rose bengal test, a very sensitive test of liver function, can be analyzed graphically to yield three quantities —uptake halftime, excretion halftime and fractional liver blood volume. The values in various liver diseases seem to be characteristic of the disease and to indicate whether cellular damage or obstruction predominates.

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Postictal Recovery in Normal and Diphenylhydantoin (Dilantin)-Treated Mice.* (22762)

David H. Tedeschi,† Ewart A. Swinyard, and Louis S. Goodman

Departments of Pharmacology, University of Utah College of Pharmacy and College of Medicine, Salt Lake City

It is well known that supramaximal electroshock stimulation of experimental animals evokes seizures characterized by 3 components: tonic hindleg flexion, tonic hindleg extension, and terminal generalized clonus. Alteration in the severity of this seizure, by drugs or experimental procedures, may be recognized by changes in duration of the tonic components of the seizure. It was shown(1) in mice that relatively *minor* changes in seizure severity can be detected by changes in duration of tonic flexion, whereas *major* changes in seizure severity can be recognized by changes in duration of both the flexor and the extensor tonic components. These criteria for changes in seizure severity have now been employed to investigate the postictal recovery process in normal and diphenylhydantoin (Dilantin)-treated mice. The results of this investigation provide the basis for this report.

Methods. A population sample of 160 adult male albino mice obtained from the Carworth Farms (CF #1 strain) was randomly divided into 2 groups (test and control) of 80 mice each. Each animal of the test group was given orally 11.0 mg/kg of diphenylhydantoin sodium (Dilantin Sodium) suspended in 10% acacia mucilage, *i.e.*, approximately an ED_5 as previously determined by the maximal electroshock seizure (MES) test; the control group was given an equivalent volume of 10% acacia mucilage. Three hours later, at the time of peak anticonvulsant effect of Dilantin by the MES test, groups of 8-10 mice (test and control) were given an initial electroshock of 50 mA (0.2-sec. duration, 60-cycle a.c., corneal electrodes) and the pattern of the "first" seizure was recorded. At various time intervals after the initial shock, a second electroshock of the same intensity was administered and the pattern of the "second" seizure was recorded. Any one animal, however, received only 2 electroshocks. The time interval between electroshocks varied from 180 to 540 seconds for Dilantin-treated mice and from 40 to 200 seconds for control mice. The mean durations of hindleg tonic flexion and extension, terminal generalized clonus, and total seizure for both "first" and "second" convulsions were calculated as follows: the durations of the individual components were summed and the values obtained divided by the total number of mice per group. In addition, the standard deviations of the means were calculated. The endpoints for these seizure components have been described previously(1). The time (RT_{50}) required for 50% of mice to recover the ability to exhibit a second tonic-clonic seizure, regardless of duration of components, after a first maximal convulsion was determined by the graphic log-probit method of Litchfield and Wilcoxon(2).

Results. The mean durations of the various components of "first" seizures and of "second" seizures elicited at the indicated times in normal mice are shown in Table I. It may be seen from the table that the mean durations of the various components and the total duration of "first" seizures were remarkably uniform, and there was no significant difference in the values obtained in the various groups of normal mice. As might be expected, the character and the duration of the "second" seizure are crucially dependent on the time, in the postictal period, of application of the second electroshock. The longer this time is extended, the more the "second" resembles the "first" seizure. Whereas, at 200 seconds, the mean tonic flexion duration of the "second" seizure was still significantly

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† University of Utah Research Fellow, 1954-1955. Present address: Smith, Kline and French Laboratories, Philadelphia

TABLE I. Mean Durations of "First" and "Second" Seizure Components in Normal Mice.

Time of application of 2nd electroshock (sec.)	Mean duration of seizure components, seconds†							
	Tonic hindleg						Total duration	
	Flexion		Extension		Clonus			
	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure
40	1.6 ± .1	6.9 ± .4	13.8 ± .7	0	6.4 ± .7	8.6 ± .7	21.8 ± .7	15.5 ± .9
60	1.6 ± .4	8.2 ± .7	13.8 ± .4	1.4 ± .9	6.4 ± .6	11.0 ± .6	21.9 ± .6	20.6 ± .6*
80	1.5 ± .2	5.9 ± 1.1	14.0 ± .5	4.9 ± 1.3	7.0 ± .6	8.4 ± .6*	22.5 ± .9	19.2 ± 1.7*
100	1.8 ± .2	5.0 ± .9	14.1 ± .3	7.2 ± 1.8	6.7 ± .3	7.9 ± .7*	22.5 ± .7	20.1 ± 1.0*
120	1.4 ± .1	2.7 ± .3	15.7 ± .7	12.6 ± .8	7.8 ± .3	9.1 ± .8*	24.9 ± .8	24.4 ± 1.2*
140	1.6 ± .1	2.8 ± .2	14.2 ± .4	11.1 ± .9	7.4 ± .2	7.5 ± .8*	23.1 ± .5	21.4 ± .9*
160	1.6 ± .1	2.6 ± .2	14.1 ± .5	13.5 ± 1.0*	7.0 ± .8	8.6 ± 1.0*	22.7 ± 1.0	23.6 ± 1.5*
180	1.6 ± .1	2.2 ± .2	14.1 ± .8	14.6 ± .9*	7.9 ± .8	8.8 ± .9*	22.8 ± .5	24.7 ± 1.1*
200	1.5 ± .1	2.3 ± .1	14.7 ± .6	14.1 ± 1.1*	7.3 ± .7	7.2 ± .8*	23.5 ± .7	23.6 ± .9*

* Not significantly different from mean value of the first seizure ($P > .05$).
† Sum of the durations of individual components divided by total No. of mice per group ± standard deviation of the mean.

longer than that of the "first" seizure, the mean durations of tonic extension, terminal clonus and total convulsion of "second" seizures were not significantly different from those of the control values after 160, 80, and 60 seconds, respectively. Thus, the order in which the durations of the various components of the "second" seizure returned to control values was as follows: total seizure duration, terminal clonus, tonic extension, and tonic flexion.

The mean durations of the various components of "first" and "second" seizures elicited at the indicated times in Dilantin-treated mice are shown in Table II. As shown in the table, there was no significant difference in the "first" seizure values obtained in groups of Dilantin-treated mice. The mean durations of tonic flexion, tonic extension, terminal clonus, and total convulsion of "second" seizures were not significantly different from their appropriate control values after 540, 400, 320, and 180 seconds, respectively. Thus, the order in which the durations of the various components of the "second" seizure returned to control values was the same as observed for untreated mice, *i.e.*, total seizure duration, terminal clonus, tonic extension, and tonic flexion.

The RT_{50} and 95% confidence limits for control mice were 75 (65-86) seconds, whereas those for Dilantin-treated animals were 320 (260-394) seconds. The Dilantin-induced increase in RT_{50} was highly significant ($P < .001$), and amounted to more than 300%.

Discussion. The data presented indicate that the pattern of a "second" maximal seizure elicited after a "first" depends on the time interval between the two seizures and

TABLE II. Mean Durations of "First" and "Second" Seizure Components in Dilantin-Treated Mice.

Time of application of 2nd electroshock (sec.)	Mean duration of seizure components, seconds†							
	Tonic hindleg						Total duration	
	Flexion		Extension		Clonus			
	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure
180	2.8 ± .2	7.8 ± .8	9.6 ± .7	1.3 ± .7	8.8 ± .8	11.3 ± 1.1	21.0 ± .9	22.8 ± .9*
280	2.9 ± .1	6.8 ± 1.0	9.2 ± .9	2.6 ± 1.3	7.8 ± .8	12.3 ± 1.0	19.7 ± 1.4	22.8 ± .9*
320	3.1 ± .3	6.3 ± 1.0	8.7 ± .8	4.8 ± 1.5	8.6 ± .6	9.0 ± .3*	20.5 ± .4	20.1 ± .9*
360	2.4 ± .2	6.0 ± 1.1	12.7 ± .8	6.9 ± 1.9	8.5 ± .9	11.1 ± 1.4*	23.6 ± 1.0	24.0 ± 1.0*
400	2.9 ± .3	5.4 ± .8	10.9 ± 1.0	7.2 ± 1.6*	7.5 ± .9	10.0 ± .7*	21.5 ± .6	22.6 ± .7*
480	3.2 ± .5	5.3 ± .8	10.2 ± .9	6.9 ± 1.6*	9.2 ± .7	11.6 ± 1.5*	22.3 ± .7	23.7 ± 1.2*
540	2.4 ± .3	3.5 ± .5*	12.0 ± 1.2	12.3 ± 1.4*	9.5 ± .6	8.9 ± .8*	24.0 ± .9	24.7 ± 1.1*

* Not significantly different from mean value of the first seizure ($P > .05$).
† Sum of the durations of individual components divided by total No. of mice per group ± standard deviation of the mean.

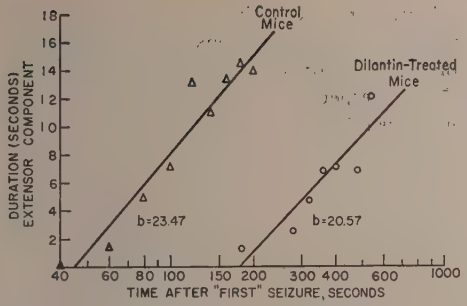


FIG. 1. Rate of recovery of hindleg tonic-extensor component in control and Dilantin-treated mice. Time interval in sec. (logarithmic scale) between "first" and "second" seizures is shown on abscissa and duration of extensor component in sec. (linear scale) is shown on ordinate. Values obtained in control mice are plotted with triangles; those obtained in Dilantin-treated mice, with circles. The continuous lines were fitted to the plotted points by the method of least square. The letter b indicates the calculated slope of the line. See text for discussion.

that the total duration of a maximal electroshock seizure is the sum of the durations of the individual seizure components. Dilantin prolonged by more than 170% the time necessary for "second" seizures to return to "first" seizure values and induced a 325% increase in RT_{50}. Previous studies from this laboratory indicate that Dilantin also increases RT_{50} in rats(3).

By what mechanism does Dilantin prolong recovery time? Interesting information on this question is revealed by plotting the duration of the hindleg tonic extensor component of "second" seizures elicited in normal and Dilantin-treated mice against the time interval between seizures. This plot is shown in Fig. 1 from which it can be seen that recovery time, as measured in these experiments, is composed of two factors: the recovery time *per se* (indicated by the slopes of the two regression lines) and the latent period before recovery of the tonic-extensor component starts (indicated by the points of intercept of the regression lines and abscissa). Since there is no significant difference in the slopes of the recovery regression lines for control and Dilantin-treated mice and since the time before recovery starts is 45 seconds in control mice and 175 seconds in Dilantin-treated mice, it would appear that the *rate* of recovery of tonic extensor ability is unaffected by this drug and that it acts primarily to prolong the latent period before recovery of the tonic-extensor component starts. This interpretation is supported by the work of Esplin who demonstrated‡ on cat spinal cord preparations that Dilantin had little effect on the level of resting excitability, as judged by transmission of an isolated impulse, but deepens the depression following an impulse.

In recent years, we have directed considerable attention to the effects of various physiological vicissitudes, drugs, etc. on the RT_{50} for maximal electroshock seizures(1,3, 4,5); however, the data presented in Tables I and II represent our first precise measurement of the time for recovery of "second" maximal seizures as related to "first" seizure values. What percentage of CNS reactivity has been regained at the time that 50% of animals again show some detectable tonic-extensor component? The return of CNS reactivity is logarithmic; hence, a definitive answer to this question can not be derived from the data presented. Nevertheless, an interesting correlation can be revealed by comparing recovery of the tonic-extensor component and RT_{50}. It may be determined from the tables that the mean durations of the tonic-extensor component of "first" seizures are 14.27 and 10.47 seconds in normal and Dilantin-treated mice, respectively. By reference to Fig. 1, it may be seen that the times for recovery of one-half the full tonic-extensor durations are 93 and 320 seconds in normal and Dilantin-treated mice, respectively. Since the respective RT_{50}s were determined to be 75 (65-86) and 320 (260-394) seconds, it may be concluded that RT_{50} is approximately the time for recovery of 50% of the *full* tonic-extensor component. Because of the comparative ease of its determination and calculation, the RT_{50} is a more useful and accurate endpoint for the measurement of recovery of CNS reactivity than is full recovery of the tonic-extensor component.

Summary. In order to study postictal recovery, groups of normal and Dilantin-treated mice were given a supramaximal electroshock

‡ *Fed. Proc.*, 1955, v14.

and the durations of the hindleg components of the "first" seizure recorded. At various time intervals after the "first" seizure, a "second" supramaximal electroshock was given and the durations of the hindleg components of the "second" seizure recorded. This procedure was repeated, with longer time intervals between seizures, until the observed durations of the "second" seizure components were not significantly different from their "first" seizure values. In addition, the time (RT_{50}) required for 50% of animals to recover ability to exhibit a second tonic-clonic seizure after a first was determined. The data obtained may be summarized as follows: The order in which the durations of the various components and the total duration of the "second" seizure returned to their "first" seizure values were the same in both groups: total seizure duration, terminal clonus, tonic extension, and tonic flexion. The total duration of a maximal seizure is relatively independent of marked changes in duration of the individual seizure components. Dilantin prolonged the recovery time of terminal clonus, tonic extension, and tonic flexion by approximately 300%, 150%, and 170%, respectively. In addition, Dilantin prolonged RT_{50} by 325%. Evidence is presented which suggests that Dilantin acts to prolong the latent period before recovery of the tonic-extensor component starts, and that the recovery process *per se,* once initiated, is relatively unaffected by the drug.

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Dehydrogenase Activity of Rat Epiphyseal Cartilage.* (22763)

RICHARD H. FOLLIS, JR. AND P. L. MELANOTTE†

Armed Forces Institute of Pathology, Washington 25, D.C.

Some years ago dehydrogenase activity was demonstrated in epiphyseal cartilage, using succinate and citrate as substrates and the decolorization of methylene blue as an indication of enzyme action(1). This technic does not allow for any precise localization of the enzymes in the cell. Hence, the application of newer technics utilizing tetrazolium compounds was undertaken, since so little is known about the metabolic activities of growing cartilage.

Materials and methods. Young (40-60 g) rats were killed by a blow on the head. After the lower extremity was dissected of muscle, free hand sections, as thin as possible, were made with a razor through the epiphyseal cartilages of the upper tibia or lower femur. Such slices were washed briefly in saline to remove any blood and were placed in depression slides containing 4 drops of .2M phosphate

TABLE I. Degree of Tetrazolium Reduction in One Hour with Different Substrates.

Control	0
Succinate	+++
Succinate and malonate	0
Citrate	++
Isocitrate	+++
Lactate	+++
Malate	+++
Pyruvate	0
Glucose	0
Glutamate	0
Oxalacetate	+
Xanthine	0

* Aided in part by grant from American Cancer Society, upon recommendation of Committee on Growth.

† Damon Runyon Fellow, 1955-56.

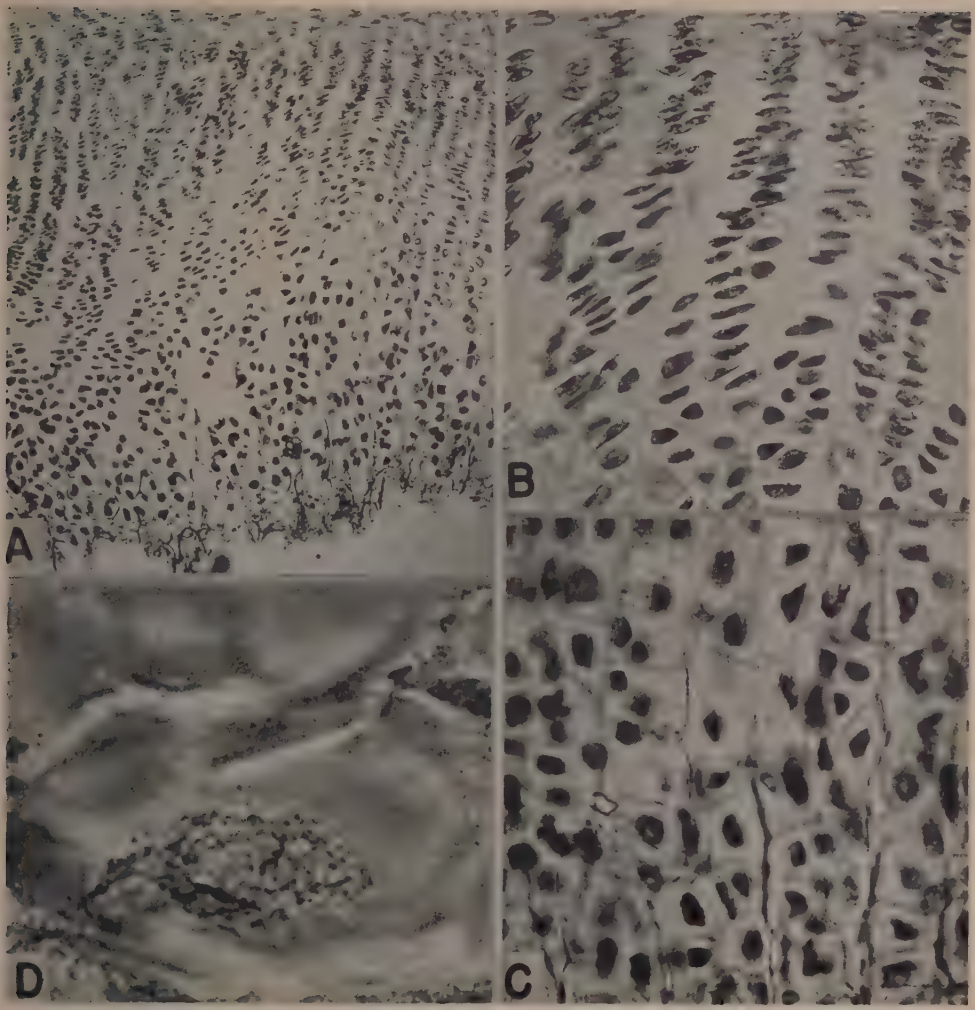


FIG. 1. Microphotographs of rat epiphyseal cartilage slices incubated with buffered sodium succinate and neotetrazolium chloride. A. Entire width of epiphyseal cartilage. ×75 (A.F.I.P. neg. 56-5005). B. Higher power of zone of proliferating cartilage cells. ×260 (A.F.I.P. neg. 56-5006). C. Higher power of zone of hypertrophic cells. ×260 (A.F.I.P. neg. 56-5007). D. High power of single cartilage cell to show rods and granules of formazan about non-staining nucleus. ×1400 (A.F.I.P. neg. 56-15407).

buffer of pH 7.4, 2 drops of .2M substrate in phosphate buffer and 3 drops of neotetrazolium chloride (0.1%) in phosphate buffer. Coverslips were applied, care being taken to exclude air; the preparations were incubated at 37°C. The following substrates were employed; glucose, succinate, malonate, malate, isocitrate, oxalactate, pyruvate, lactate, glutamate, xanthine, alpha-ketoglutarate and alanine. Control slices were incubated with 2 drops of buffer substituting for substrate. The preparations were observed under the microscope every half hour, generally for at least 2 hours.

Results. The distribution of enzyme activity with succinate as a substrate will first be described. Purplish granules of formazan pigment began to appear after one half hour of

incubation, reaching a maximum in 2 hours. Under the low power of the microscope such pigment deposits seemed to delineate the nuclei of the cartilage cells. Under higher power, however, these structures were seen to remain unstained; the granules and short rods of pigment were grouped around an unstained area (the nucleus) in the center of the cell. A wide clear zone was present between this pigment and cell membrane. Formazan pigment was found in the cartilage cells at all stages of development. During the time of observation no formazan pigment developed in the slices incubated with malonate or without substrate.

Variations in degree of intensity of the strain depending on the substrate used are shown in Table I. Those leading to the most pigment formation in the shortest period of time were succinate, isocitrate, citrate, lactate, and malate.

Discussion. These histochemical observations confirm the presence of dehydrogenase activity of epiphyseal cartilage described earlier(1) and amplify the few biochemical studies which have been carried out on articular(2) and epiphyseal(3,4) cartilages.

The epiphyseal and costal cartilages are unique in that they display all stages of differentiation and growth at a time when other tissues in the organism have matured. The needs of the epiphyseal cartilages for energy to grow must be great when it is realized that a rat's lower femur increases .18 mm in length per day(5). Hence, it is not surprising to find evidence for the active metabolism of certain compounds involved in the Krebs cycle. Exactly how such data may fit into the total metabolism of cartilage remains to be elucidated. These observations would appear to have no bearing on the calcification mechanism since activity was found in all strata of the epiphyseal cartilage. However, they provide some understanding for the presence of citric acid in cartilage(6) though they do not indicate what proportion may be utilized in the citric acid cycle and what amounts may be purely fortuitous, as in bone(7).

Summary. Utilizing the tetrazolium technic, intracellular dehydrogenase activity has been localized in epiphyseal cartilage cells. The most favorabe substrates appear to be succinate, isocitrate, citrate, lactate and malate.

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Effect of Aminonucleoside on Ribonucleic Acid Content of Mouse Mammary Adenocarcinoma *in vivo*. (22764)

J. C. VAN METER, E. H. SNEDEKER, L. R. MOSER, AND J. J. OLESON

Research Division, American Cyanamid Co., Pearl River, N. Y.

Puromycin (6-dimethylamino-9-[3′-deoxy-3′ (p-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl]purine), an antibiotic isolated by Porter *et al.*(1), and structurally characterized by Waller *et al.*(2) has been shown by Troy *et al.*(3) to inhibit the growth of certain experimental tumors. It has further been shown by Oleson *et al.*(4) that the carcinostatic activity for mouse mammary adenocarcinoma resides in the aminonucleoside portion of puromycin. The structure of the aminonucleoside suggests that its antitumor activity could involve nucleic acid metabolism. To test this hypothesis, C3H mammary adenocarcinomas were analyzed for RNA content and composition, and compared to the same tumor

TABLE I. Purine-Bound Ribose Content of C3H Mammary Adenocarcinomas.

Group	Treatment	Mean tumor wt, mg*	μM PBR per g tissue†
1	0.2 ml 0.9% NaCl in-	143	5.0
2	traper. twice daily	352	6.2
3	for 2½ days	675	6.8
4		1800	6.3
5		2500	5.9
	Mean	1094	5.84‡
6	3 mg 2484L in 0.9%	220	2.8
7	NaCl intraper. twice	362	3.5
8	per day for 2½ days	625	3.8
9		1400	4.7
10		2320	4.0
	Mean	985	3.76‡

* Mean wt of similar sized tumors from 2 to 15 backcrossed C3H mice depending on tumor size.

† Micromoles of purine-bound ribose/g wet tissue.

‡ Difference between means of purine-bound ribose of the 2 treatments is statistically significant since $P < 0.008$ by analysis of variance.

from mice which had been treated with 6-dimethylamino-9- [3′-deoxy-3′-amino-β-D-ribofuranosyl]purine (2484L).

Methods. In order that an appropriate comparison might be made, the established tumors on backcrossed C3H mice were so selected that the control and 2484L treated tumors would be of approximately equal size at the end of the treatment period. Mice bearing much larger tumors were selected for 2484L treatment than those intended for no treatment. The aminonucleoside (2484L), pre pared by the method of Baker *et al.*(5) was injected intraperitoneally into the mice in five 1.5 mg doses in 0.9% NaCl at intervals of about 12 hours. At the end of the treatment period, the mice were decapitated and the tumors rapidly excised and frozen in dry ice. The frozen tumors were homogenized in cold 6% $HClO_4$ and the ribonucleic acid extracted by a modified Schmidt-Thannhauser-Schneider procedure(6). The RNA was estimated as purine-bound ribose by the orcinol reaction (7). A portion of the ribonucleotide containing extract was paper chromatographed and the component monoribonucleotides estimated by the method of Elson *et al.*(8).

Results. The RNA (purine-bound ribose) content of 2484L treated tumors was significantly less than that of untreated controls

TABLE II. Ribonucleotide Distribution in C3H Mammary Adenocarcinomas.

Group*	Treatment	Mean tumor wt, mg	Adenylic acid		Cytidylic acid		Guanylic acid		Uridylic acid		Total μM mononucleotide/g	Ratio, μM purine to μM pyrimidine
			μM/g†	Base ratio‡	μM/g†	Base ratio‡	μM/g†	Base ratio‡	μM/g†	Base ratio‡		
1	.9% NaCl	143	2.30	1.00	3.33	1.45	4.00	1.74	1.59	.69	11.11	1.29
4		1800	2.63	1.00	3.92	1.50	4.34	1.65	2.36	.90	13.25	1.11
6	3 mg 2484L	220	1.20	1.00	1.64	1.37	2.08	1.73	.79	.66	5.71	1.36
9		1400	1.92	1.00	2.84	1.48	3.40	1.77	1.72	.90	9.90	1.17

* Group No. corresponds to tumor group in Table I.

† Micromoles/g wet tissue. All values are means of 3 determinations.

‡ Ratio of μM of base to that of adenine.

(Table I). The nucleotide composition of the RNA fraction of the tumors revealed no gross difference in base ratios (Table II). A comparison of the total ribonucleotide contents of these groups confirms the lower RNA concentration of the 2484L treated tumors. The effect of 2484L treatment on tumor RNA cannot be ascribed to hydration, since it was found that the total nitrogen per gram wet weight of similarly treated and control groups did not differ significantly. These results suggest that 2484L causes an inhibition of synthesis (or an acceleration of degradation) of RNA in C3H mammary carcinoma. Ledoux and Revell(9) have shown that the decrease in growth rate of an ascites tumor (Landschütz) is associated with a parallel decrease in RNA content under normal conditions of growth. Thus the inhibition of growth of this tumor by 2484L may be associated with the decrease in RNA content.

Summary. (1) The RNA content of 2484L treated C3H mammary adinocarcinomas was shown to be lower than that of untreated controls. (2) The nucleotide composition of both treated and control tumors appeared to be similar.

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Anaerobic Glycolysis of Brain Cortex Slices in Hyperthyroid and Control Rats.* (22765)

M. A. SPIRTES AND D. SCHWARTZ

Division of Pharmacology, Hahnemann Medical College, Philadelphia, Pa.

The primary site of action of the thyroid hormone in the intermediary metabolic scheme cannot yet be considered as definitely established. An early theory first enunciated by Haffner shortly after the advent of the Warburg metabolic techniques, was that the thyroid hormone primarily affects a step involved in anaerobic glycolysis(1). This was disputed by McEachern who claimed that anaerobic glycolysis was not increased in the livers of rats made hyperthyroid, altho slices of such livers invariably showed increased oxygen consumption when compared with normal control liver-slice values(2). His data confirmed similar earlier work of Dresel(3). Furthermore Anselmino *et al.* had early claimed that spleen slices from hyperthyroid rats failed to show a rise in either oxygen consumption or anaerobic glycolysis in a comparison with normal controls(4). However, in view of a recent statement by Goldstein that anaerobic glycolysis is increased in hyperthyroid rats(5), it was decided to reinvestigate the problem of the relationship of thyroxin to glycolysis beginning with the anaerobic type and brain cortex slices for a reason to be discussed later.

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Methods. L-thyroxine is dissolved in a

TABLE I. Anaerobic Glycolysis of Brain Slices from Control and Hyperthyroid Rats.

Type of animal	No. of animals	No. of values	Anaerobic glycolysis expressed as $Q_{CO_2}^{N_2}$ *
Normal control rats	12	24	18.9 ± 2.34†
Hyperthyroid "	8	24	16.7 ± 3.66

* See text.
† Stand. dev. of mean.

minimal amount of 0.05N NaOH (1 mg in 0.1 cc of 0.05N NaOH). Two mg of such a thyroxine solution are injected subcutaneously into white Wistar rats weighing 100-200 g and this is followed by similar injections of 1 mg daily for 2-4 days, the thyroxine solution being made up fresh daily. The treated rats and untreated litter mates were fed *ad lib* with Purina rat checkers and water. All rats were decapitated for the experimental runs and cerebral cortex slices prepared from each hemisphere manually with a double-edged razor blade held by a Kelly Clamp. One or two slices, less than 0.5 mm thick, were placed into Warburg flasks each containing 3 cc of Krebs-Ringer-Bicarbonate-0.1% glucose solution(6). The vessels were gassed for 5 minutes with a 5% CO_2-95% N_2 gas mixture and placed in a Warburg bath at 37°C. After an 8-minute temperature equilibration period, CO_2 release from the bicarbonate buffer due to anaerobic glycolysis was measured every 15 minutes for 1 hour.

Results. The results are expressed in Table I as cmm CO_2 released per mg dry weight of brain cortex per hour ($Q_{CO_2}^{N_2}$), the slices having been dried for 24 hours at 100°C.

Discussion. Since anaerobic glycolysis in the liver is extremely low and possibly even rudimentary, the fact that it does not increase in liver slices(2-4) from hyperthyroid animals might be considered of doubtful significance. Brain cortex slices, like spleen, however, could be of value as a test for a primary thyroid hormone effect on glycolysis since they show no rise in oxygen consumption in the hyperthyroid state, but do possess a high rate of anaerobic glycolysis normally. Thus a rise in anaerobic glycolysis in hyperthyroid brain slices could not be considered as secondary to a rise in O_2 consumption, since the latter does not occur(7). The fact that such brain slices from hyperthyroid rats do *not* exhibit a higher rate of anaerobic glycolysis than similar slices from normal rats corroborates similar findings with spleen slices reported by Anselmino *et al.*(4). These results combined with those of Dresel(3) and McEachern(4) make the hypothesis of a primary effect of the thyroid hormone on anaerobic glycolysis very unlikely, considering especially that positive, physiological effects on the brain in different thyroid states have been reported(8) and are known clinically. The possibility must still be considered, however, that all the positive brain findings are secondary and due to influence of the thyroid gland upon other glands of internal secretion. If such were the case, then it is possible that the thyroid hormone has no direct effect on any phase of brain metabolism.

Conclusion. Anaerobic glycolysis is *not* increased in brain slices from rats injected with 4-8 mg of L-thyroxine, when compared to similar values from normal control rats.

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Development of Resistance and Cross-Resistance *in vitro* to Erythromycin, Carbomycin, Spiramycin, Oleandomycin and Streptogramin.* (22766)

Wilfred F. Jones, Jr., Roger L. Nichols, and Maxwell Finland†

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and Department of Medicine, Harvard Medical School, Boston, Mass.

Among the major problems in the field of infectious diseases today, particularly in hospitals, is the occurrence, with increasing frequency, of strains of *Micrococcus pyogenes*, var. *aureus*, resistant to many antibiotics that are most commonly used. This has been accompanied by a serious increase in incidence and severity of infections due to such organisms and in difficulties encountered with the use of antibiotics in their management (1,2).

Among recent antibiotics, the one most active against the staphylococcus has been erythromycin, but this organism has been shown to acquire resistance quite rapidly as a result of exposure to erythromycin, both *in vitro* and during the therapy of patients (1,3, 4). Carbomycin, another antibiotic subsequently made available, was also shown to develop almost complete cross-resistance to erythromycin *in vitro* (5-7). Additional antibiotics with considerable *in vitro* activity against *M. aureus* have been developed more recently, and some of them have already received some clinical trials. The present paper presents results of studies on development of resistance and cross-resistance of *M. aureus in vitro* to 3 of these new agents, namely spiramycin (8-14), oleandomycin (PA-105) (15, 16) and streptogramin ("Antibiotic 900") (17), and these, in turn, were tested for cross-resistance to erythromycin and carbomycin.‡ The effect of exposures to these 5 antibiotics on susceptibility of the strains to almost all commonly used antibiotics and to some additional new ones was also tested.

Materials and methods. Four strains of *M. aureus* were used, 2 of them (strains A and B) were highly sensitive to penicillin and the tetracyclines, whereas the other 2 (strains C and D) were highly resistant to them. All 4 were originally inhibited by moderately low concentrations of each of the 5 antibiotics under study. The strains were grown on the surface of a series of plates containing heart infusion agar (Difco, pH 7.2 ±) in which graded concentrations of the antibiotics were incorporated. The original inoculum was derived from a freshly grown overnight culture of the strain in broth and this was streaked on to the agar with a 2 mm loop. The confluent growth from the surface of the agar containing the greatest concentration of antibiotic on which growth was similar to that occurring without antibiotic was used for the next passage to a similar series of plates containing the same antibiotic; this growth was scraped off with a larger loop and emulsified in 0.5 ml of broth and the resulting suspension used for the transfer. The process was repeated until full growth occurred on plates containing 400 μg/ml, the largest concentration used in this study, and was then usually transferred a number of additional times on this concentration of the agent. After each 3 subcultures on one of the antibiotics, the inoculum for the next subculture was also used for tests on similar series of plates containing the other 4 antibiotics in order to determine the extent of the cross-resistance that had developed. At the completion of the study, all of the parent cultures, which had been subcultured in parallel on antibiotic-free agar, and those that had been serially exposed to each of the 5 antibiotics were tested, in addition, for sensitivity to 10 antibiotics, including 6 that are in com-

* Aided by grant from National Institutes of Health.

† With technical assistance of Clare Wilcox and Ann Najarian.

‡ Spiramycin and streptogramin were furnished by Sharp & Dohme Division, Merck and Co., the Oleandomycin was supplied by Parke-Davis Co. and also by Chas. Pfizer and Co., the latter also furnished the carbomycin (Magnamycin), and the erythromycin (Ilotycin) was obtained from Eli Lilly and Co.

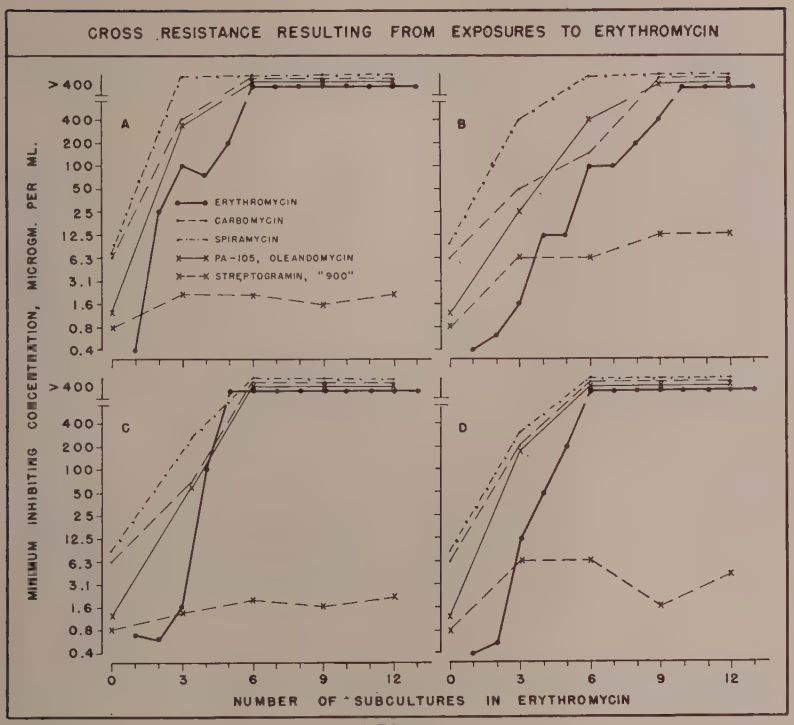


FIG. 1.

mon use and 4 that are currently under investigation.

Results. The minimum inhibiting concentration (M.I.C.) of erythromycin for each of the 4 strains§ after each successive transfer on that antibiotic, and the susceptibility to the other 4 antibiotics of these strains after each third subculture is shown in Fig. 1. All 4 strains were originally inhibited by 0.4 μg/ml of erythromycin. Each of them developed resistance to the erythromycin very rapidly in the presence of that antibiotic. Strain C grew uninhibited on agar containing 400 μg/ml of that antibiotic after 5 subcultures, Strains A and D after 6 and Strain B after 10 such exposures. Cross-resistance to 3 of the other antibiotics developed at about the same rate, as evidenced by the similar slope of the curves in the figures. The M.I.C. of each of the parent strains for these 3 antibiotics (spiramycin, carbomycin and oleandomycin) was 2 to 8 times that for erythromycin, and the number of transfers required to render the strains resistant to 400 μg/ml of these agents was somewhat greater. Streptogramin was the exception; only a slight decrease in susceptibility of the strains to this antibiotic occurred after the first few exposures to erythromycin but there was no further increase in cross-resistance after further transfers on erythromycin.

Resistance of the same strains to carbomycin resulting from the repeated exposures to that antibiotic developed at a variable rate in the different strains, most rapidly in Strain C (Fig. 2). Cross-resistance to the other antibiotics resulting from these exposures to carbomycin, varied in a similar manner, and developed either at the same rate or at somewhat slower rate. Cross-resistance to spiramycin occurred with particular rapidity, at a rate equal to or even exceeding that against the homologous antibiotic; however, the par-

§ The strains are designated A,B,C, and D corresponding to the letters shown in the panels of the text figures.

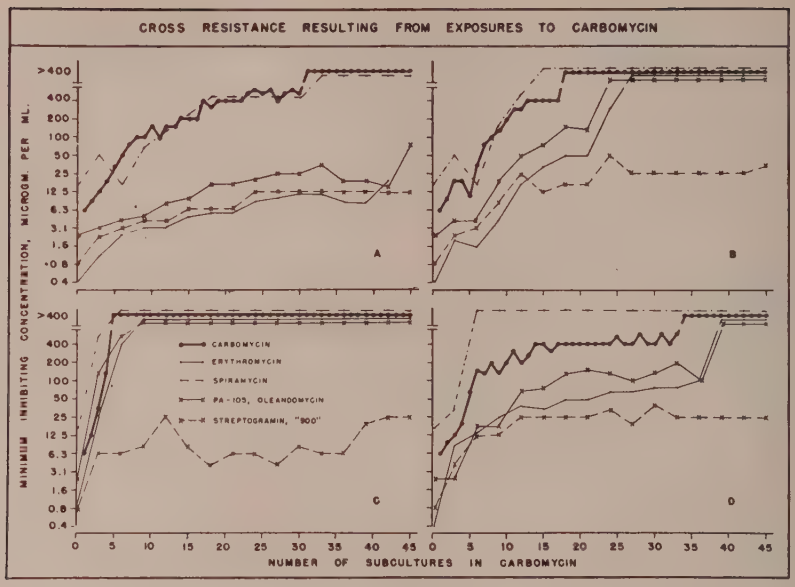


FIG. 2.

ent strains were also somewhat less susceptible to spiramycin than to carbomycin. The reverse was true with respect to the other 3 antibiotics; the parent strains were somewhat more susceptible and they developed cross-resistance at the same or somewhat slower rate, and more subcultures were required before the strains acquired cross-resistance to 400 μg/ml. For Strain A this degree of resistance was not reached after a total of 45 subcultures, or 15 transfers after this strain had acquired that degree of resistance to both carbomycin and spiramycin. Cross-resistance against streptogramin occurred to a somewhat greater extent than after exposure to erythromycin and this tended to level off after the first few exposures to carbomycin just as it did after the exposures to erythromycin.

Exposures to spiramycin (Fig. 3) resulted in the development of resistance to the homologous antibiotic and cross-resistance to the others at about the same rate as was observed with erythromycin. However, only Strain A showed any significant cross-resistance to streptogramin and this appeared to be transient.

Oleandomycin also induced resistance to the homologous agent and to all of the others, except streptogramin, quite rapidly and uniformly. There was no change whatever in the M.I.C. of the 4 strains to streptogramin during the 12 subcultures on oleandomycin (Fig. 4).

Exposures of the strains to streptogramin (Fig. 5), resulted in the development of resistance to both the homologous antibiotic and to the other 4 at a slower rate than was shown in the previous figures. From 32 to 43 subcultures on streptogramin were required to increase the resistance of the strains so that they grew in the presence of 400 μg/ml of that agent. Cross-resistance to the other agents developed at about the same rate and somewhat more slowly to erythromycin.

After these observations were completed, all of the parent strains which had been transferred in parallel on the same kind of agar without antibiotics, were again tested for their sensitivity to the 5 antibiotics used in the study and these, together with the strains that had been exposed and became resistant to the 5 antibiotics were tested for sensitivity to the following additional antibiotics: penicillin, streptomycin, neomycin, tetracycline, chloramphenicol and bacitracin, and also to 4 new antibiotics: bryamycin, cycloserine, albamy-

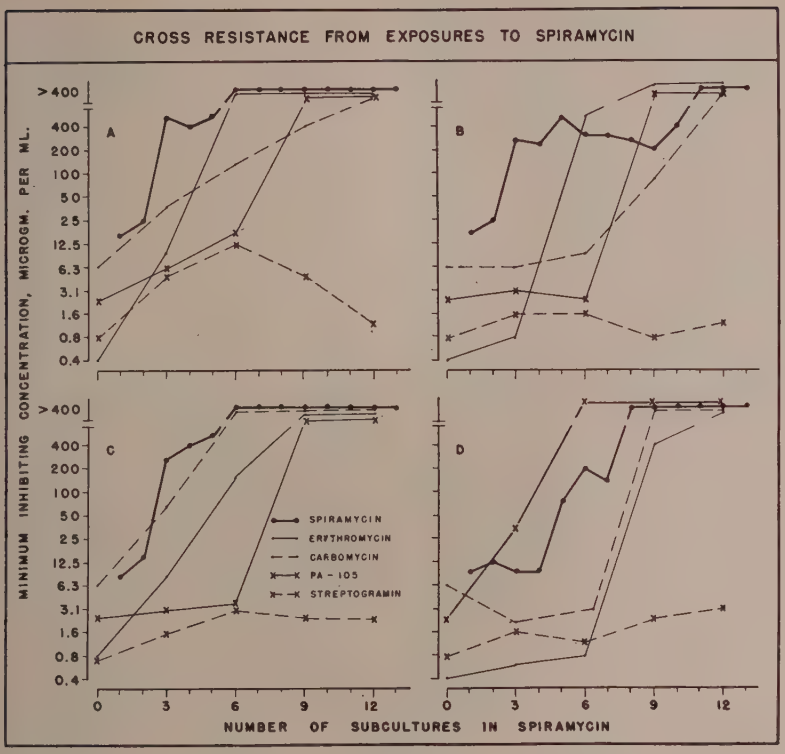


FIG. 3.

cin and vancomycin. The strains had retained their original degree of susceptibility to the 5 antibiotics during their repeated subculture on the agar without the antibiotics, and both these strains and the 5 series of resistant strains derived from them, exhibited the same susceptibility, within the limits of the method, to each of the other antibiotics. In other unpublished studies from this laboratory, strains made resistant by repeated subcultures in cycloserine, bryamin, albamycin and vancomycin showed no cross-resistance to each other, nor to any of the 5 antibiotics that were the subject of the present study, nor to the other antibiotics that are in common use. Resistance to vancomycin developed at a rate even slower than was shown here for streptogramin.

Discussion. The need for effective agents to combat the increasing number and severity of staphylococcal infections still offers a great challenge even now, with such active antibiotics as penicillin, the tetracyclines and erythromycin. These agents are still highly effective so long as the staphylococci in the lesions remain susceptible, but the nature of the staphylococcal lesion, like that in tuberculosis, is particularly favorable for the development and persistence of resistant variants. The organisms persist, often intracellularly, in necrotic and well walled-off lesions, rendering them relatively inaccessible to the full impact of systemically administered antibiotics; as a result, the staphylococci may increase in resistance and become even more difficult to eliminate. Moreover, staphylococci are readily available from the environment to contaminate superficial wounds and to spread from carriers who harbor them as saprophytes but in whom resistant variants may be selected out and then continue to be carried during antibiotic treatment or prophylaxis against other infections. This situation emphasizes the need for a highly effective

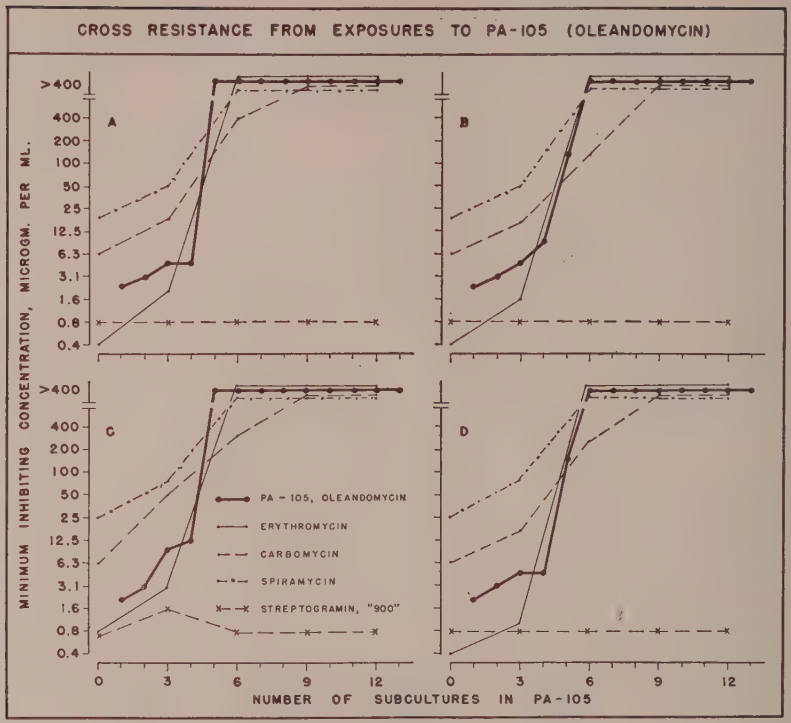

CROSS RESISTANCE FROM EXPOSURES TO PA-105 (OLEANDOMYCIN)
MINIMUM INHIBITING CONCENTRATION, MICROGM. PER ML.
>400
400
200
100
50
25
12.5
6.3
3.1
1.6
0.8
0.4
A
B
C
D
PA-105, OLEANDOMYCIN
ERYTHROMYCIN
CARBOMYCIN
SPIRAMYCIN
STREPTOGRAMIN, "900"
0 3 6 9 12
NUMBER OF SUBCULTURES IN PA-105

FIG. 4.

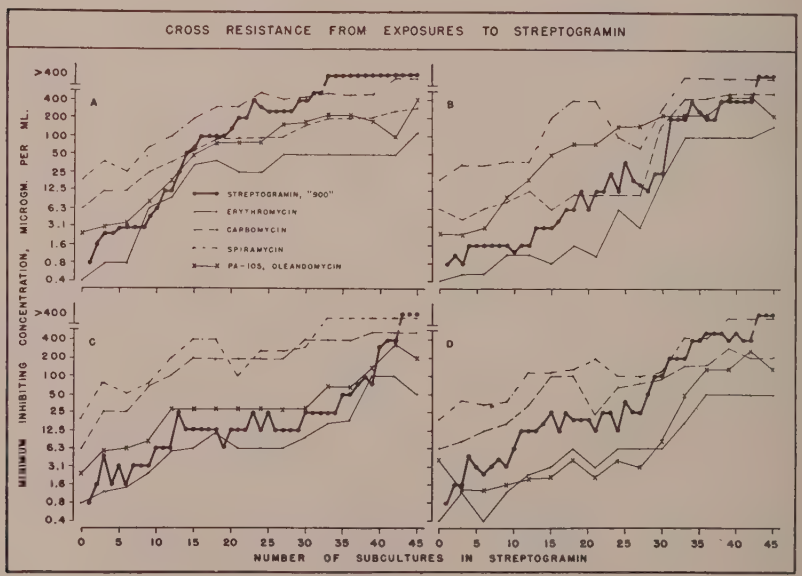

CROSS RESISTANCE FROM EXPOSURES TO STREPTOGRAMIN
MINIMUM INHIBITING CONCENTRATION, MICROGM. PER ML.
>400
400
200
100
50
25
12.5
6.3
3.1
1.6
0.8
0.4
A
B
C
D
STREPTOGRAMIN, "900"
ERYTHROMYCIN
CARBOMYCIN
SPIRAMYCIN
PA-105, OLEANDOMYCIN
0 5 10 15 20 25 30 35 40 45
NUMBER OF SUBCULTURES IN STREPTOGRAMIN

FIG. 5.

bactericidal agent, with good penetrating power, which can be used for long periods systemically, and which lacks the property of inducing resistance in staphylococci.

The present study demonstrated that at least 4 of the 5 agents that were investigated readily induced the development of resistant variants in the strains of staphylococci. There were some differences in the rate at which such resistance developed, depending on the antibiotic, and to a less extent on the strain. Moreover, cross-resistance developed at a rate comparable to that of the homologous resistance except against streptogramin; this antibiotic induced resistance more slowly than did the other 4 agents and, in turn, showed little tendency to lose its activity against the strains that had acquired resistance to those other agents. The possibility that vancomycin may be even more favorable in this respect is suggested by our own unpublished observations which corroborate those of others(18).

The data presented here were obtained *in vitro* and are valid for the conditions of the study but may not necessarily hold for all conditions of exposure of the bacteria to the antibiotics *in vitro,* and certainly may not be true *in vivo*. Ross, for example found 22 strains from patients that were resistant to erythromycin but were either sensitive, or at least moderately so to oleandomycin(19). Chabbert(10), however, found cross-resistance to erythromycin, carbomycin and spiramycin in every strain isolated from patients who had been treated with erythromycin. Observations made in this laboratory on erythromycin-resistant strains isolated from patients,‖ indicated variable degrees of cross-resistance to carbomycin and many strains of staphylococci showed none, although such cross-resistance has been observed regularly after exposures to these agents *in vitro*(5-7). However, nearly 70% of the erythromycin-resistant strains were also resistant to oleandomycin and more than one-fourth of them were resistant to spiramycin.

Summary and conclusions. Four strains of *M. aureus* were rendered resistant to erythromycin, carbomycin, oleandomycin, spiramycin and streptogramin by repeated subcultures in the presence of increasing concentrations of these antibiotics. Cross-resistance to about the same degree developed to all of these agents except to streptogramin; the latter produced homologous resistance at a slower rate than the others, but this was associated with a corresponding increase in cross-resistance to the other 4 antibiotics.

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‖ Obtained from Dr. Mark H. Lepper.

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In vivo Release of Histamine from Rabbit Blood by Reserpine. (22767)

T. Phillip Waalkes and Herbert Weissbach (Introduced by R. W. Berliner)

Laboratory of Chemical Pharmacology, National Heart Institute, N.I.H., P.H.S., U. S. Department of Health, Bethesda, Md.

Reserpine is a widely employed and useful drug in the treatment of a variety of clinical conditions, particularly hypertension and certain psychiatric disorders. One of the most common and consistent side effects of reserpine therapy in man has been nasal stuffiness. This effect may be relieved in most patients by the use of vasoconstrictive substances and in some by oral medication with antihistaminics(1). In addition, reserpine has been found to cause an increase in the volume and free acidity of gastric secretion following both oral and intravenous administration of this drug to humans(2). These secondary findings suggested the possibility that reserpine could cause the release of histamine from its body sources. Recent studies have shown that reserpine liberates serotonin (5-hydroxytryptamine) from its depots in animals(3), and from human platelets *in vivo*. Both histamine and serotonin are present in rabbit blood platelets in relatively large amounts as compared to other amines. Previous studies had indicated that reserpine incubated *in vitro* with rabbit platelets caused a release of platelet serotonin but no histamine release.

The following study was done to determine whether or not reserpine liberated histamine *in vivo* from rabbit blood.

Methods and materials. Male rabbits (1.5-2.0 kg) were given a single intravenous injection of reserpine (5 mg/kg). A control blood specimen was taken prior to the injection, and experimental samples at various intervals after the injection. All samples were drawn by cardiac puncture into siliconed glassware using heparin as anticoagulant. Both histamine and serotonin were determined chemically on whole blood. For each determination, 1 ml of whole blood was diluted to 8 ml with water, 1 ml of 10% zinc sulfate solution added and, after shaking, 0.5 ml of 1 N sodium hydroxide. Following centrifugation at high speed, a 1 ml aliquot of the supernatant was analyzed directly for serotonin by measuring its specific fluorescence in strong acid(4). Five ml of the remaining supernatant were analyzed for histamine employing modifications of the methods of McIntire *et al.* (5,6) and Lowry *et al.*(7). The details of the methods employed here, whereby both serotonin and histamine can be determined on the same supernatant after protein precipitation will be reported later. Recoveries, standards and blanks were run along with each determination. For both serotonin and histamine, recoveries were over 80%. The rabbit whole blood values obtained in this work compare favorably with those previously reported for histamine(6,8) and serotonin.*

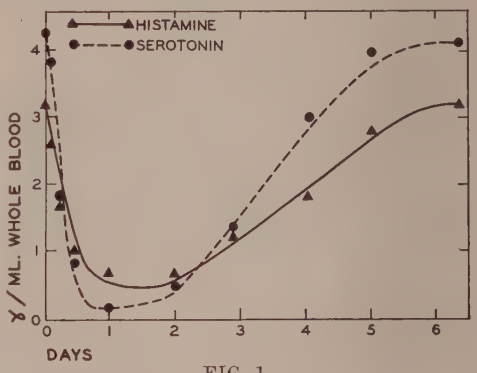


FIG. 1.

Results. Composite data from 5 animals given reserpine are shown in Fig. 1. The reduction in whole blood histamine was similar in rate and degree to the reduction in whole blood serotonin. The return of histamine and serotonin values to control levels was also parallel. The curves for each individual animal were substantially the same, reaching a minimal level at about 20 hours, remaining low until about 48 hours, and returning to normal in 5 to 7 days. The minimum histamine level at 40 hours varied from 7% to 25% of the control value in the animals. All the rabbits showed typical reserpine effects. Previous

* Unpublished observations.

TABLE I. Effect of Reserpine on Rabbit Blood, and Platelet Serotonin and Histamine.

	Serotonin		Histamine	
	Control	24 hr	Control	24 hr
Whole blood, γ/ml*	4.6	.5	3.0	.6
Platelet, γ/mg protein†	6.8	.7	3.7	.7

* Avg of 5 animals. † Avg of 2 animals.

work has shown that platelet counts are essentially the same in rabbits before and 16 hours after the intravenous injection of 5 mg/kg of reserpine.* Since in the rabbit both histamine and serotonin are found in platelets, the decrease in whole blood histamine and serotonin cannot be accounted for by a reduction in whole blood platelet count. Instead, a release from platelets is postulated with subsequent metabolism of both serotonin and histamine to account for the reduction of these constituents in whole blood.

In support of a release phenomenon to account for the drop in whole blood histamine and serotonin, platelets were isolated according to the method of Dillard *et al.*(9) from rabbit whole blood before and again 24 hours after an intravenous injection of reserpine. Both histamine and serotonin were determined and related to the amount of platelet protein(10) as shown in Table I.

Discussion. It is well substantiated that reserpine liberates serotonin from the body depots in animals, and from platelets in man. In the study presented here, histamine has been shown to be liberated from rabbit blood *in vivo*. Recently Carlsson(11) has shown epinephrin release from rabbit adrenals by reserpine, but further work* has shown that this release does not occur in a cord sectioned animal which indicates an indirect effect. These studies suggest that reserpine is capable of liberating several potent biologically active substances from blood and tissues, although its action may not necessarily be a direct one.

Histamine is known to cause the release of catechol amines(12), particularly in patients with pheochromocytomas, and in turn is released by a variety of primary amines(13,14), including epinephrine(15,16). Feldberg and Smith(17) have shown the release of histamine from tissues by serotonin using bioassay. It is therefore possible that these compounds are mutually able to release each other from blood and tissues although perhaps to a variable degree and extent. Reserpine may be capable of a primary liberation of histamine, serotonin and epinephrine, or by liberating one of these cause a secondary release of the others. That reserpine may liberate other substances also becomes a possibility. Finally, one can speculate that the therapeutic effects as well as the side effects of this drug may be due to the release of these basic compounds, their attendant interaction, and resultant loss. However, preliminary experiments indicate that reserpine does not release histamine from other animal tissues *in vivo*.

Studies on the release of histamine from tissues in animals, and blood in humans are in progress in this laboratory.

Summary. Intravenous reserpine in rabbits has been demonstrated by chemical means to release histamine from whole blood. 1. The minimal histamine levels were reached in one to 2 days with complete recovery to control values in 5 to 7 days. 2. Simultaneous serotonin determinations, revealed that the rate and degree of liberation, and the recovery of both histamine and serotonin were very similar. 3. Platelets, isolated 24 hours after reserpine, showed a marked reduction in both histamine and serotonin compared to control platelets.

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Electrophoretic Behavior of Some Animal Hemoglobins. (22768)

V. P. SYDENSTRICKER, R. OLIVER, B. M. CHANDLER, AND O. SYDENSTRICKER
Department of Internal Medicine, Medical College of Georgia, Augusta

The authors have been unable to find any report of results of paper electrophoresis of hemoglobins other than human. The present communication presents findings in 6 species of animals.

Methods. Electrophoresis was performed on "sandwich" type plate glass apparatus using Whatman 3MM paper in sheets 36 × 16 cm and 36 × 12 cm. Barbiturate buffer at pH 8.6 and M 0.025 was used in all preliminary experiments. Current was applied to 36 × 16 cm sheets at 300 volts and 5 milliamperes for 6 hours and to 36 × 12 cm sheets for 4 hours, constant temperature of 20°C was maintained. Under these conditions normal human hemoglobin (Hb. A) migrated approximately 5 cm during the run. In addition to electrophoresis alkali denaturation tests(1) and solubility tests(2) were carried out on all specimens (Table I). Rat hemoglobin was obtained from the pooled blood of adult Wistar rats killed at the end of experiments. Dog hemoglobin was from mongrel dogs used in experimental surgery. Rabbit hemoglobin was from albino rabbits used for pregnancy tests. Chicken hemoglobin was from pooled blood obtained from a poultry farm. Turtle hemoglobin was from several specimens of the common land turtle, *Gopherus polyphemus*. Alligator hemoglobin was from a single small specimen of *Alligator missippiensis*. Hemoglobin solutions were prepared in the usual manner and adjusted to approximately 5%, 3 lambda of the solution were applied to the paper.

TABLE I. Resistance to Alkali Denaturation and Solubility as COHb of Animal Hemoglobins.

Species	% alkali resistant	% soluble as carbonmonoxy-hemoglobin
Dog	0	100
Rabbit	36	"
Rat	100	"
Chicken	35	"
Alligator	0	"
Turtle	0	"

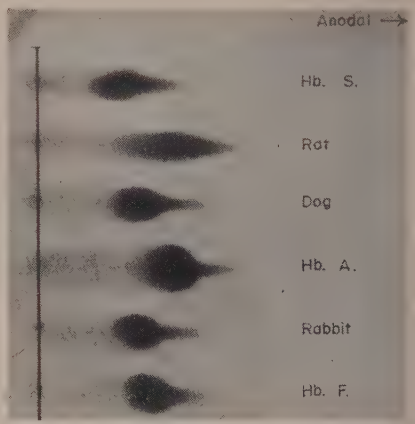


FIG. 1. Patterns of rat, dog and rabbit hemoglobins with Hb. S., Hb. A. and Hb. F. for comparison.

Results. As shown in Fig. 1, dog and rabbit hemoglobins move as homogenous compounds at a rate slightly slower than fetal hemoglobin but faster than Hb. S while rat hemoglobin produces a trailing spot suggest-

ing a mixture of hemoglobins; this we have been unable to resolve by varying the buffer or any other factors.

In Fig. 2, turtle and chicken hemoglobin behave as mixtures, these were fairly well separated by electrophoresis in phosphate buffer at pH 7.4 (Fig. 4). Alligator hemoglobin

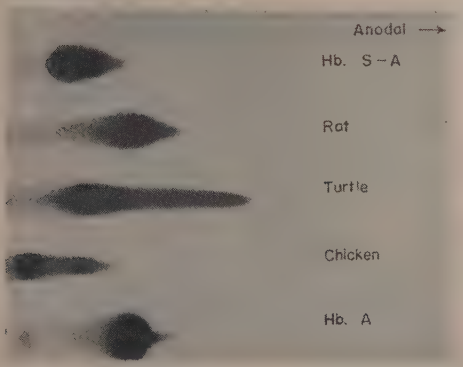


FIG. 2. Patterns of turtle and chicken hemoglobins with rat, Hb. S-A and Hb. A for comparison.

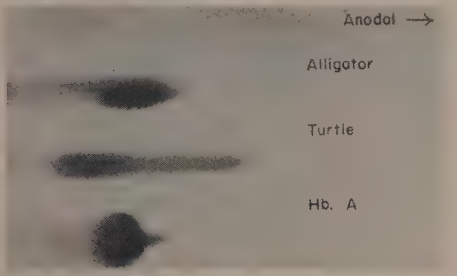


FIG. 3. Patterns of alligator and turtle hemoglobins with Hb. A for comparison.

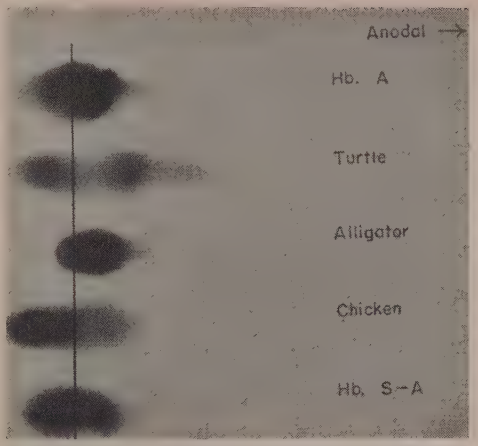


FIG. 4. Patterns of turtle, alligator and chicken hemoglobins run in phosphate buffer at pH 7.4, 150 volts, 5 milliamperes for 6 hr. Hb. A and Hb. S-A for comparison.

(Fig. 3) moves at a slightly more rapid rate than Hb. A and presents a trailing spot which does not resolve into 2 components at pH 7.4.

These observations are preliminary and incomplete in that many methods of examination of hemoglobins have not yet been employed. They are presented in the hope that they may be of interest to zoologists and perhaps phylogenists.

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Production of Fatty Livers by 4-Aminopyrazolo-(3,4-d)-Pyrimidine. Toxicological and Pathological Studies.* (22769)

JEAN SCHOLLER, FREDERICK S. PHILIPS, AND STEPHEN S. STERNBERG
(With assistance of Barbara B. Adams and Barbara Wheelock)
Sloan-Kettering Institute, Sloan-Kettering Graduate Division, Cornell University Medical College and Memorial Center for Cancer and Allied Diseases, New York City

Recently interest has been aroused in the possible use of the pyrazolopyrimidines as potential chemotherapeutic agents for the treatment of human neoplasms. Robins(1) synthesized a series of such compounds with the hope that the isomers of the naturally-occurring purines might be active. Subsequently the adenine isomer, 4-aminopyrazolo-(3,4-d)-pyrimidine (4-APP), was shown by Skipper (2) to be the most active of the series in inhibiting the mouse mammary adenocarcinoma 755 and in increasing the life span of mice with L1210 or L5178 leukemia. The results indicated that 4-APP might be worthy of clinical trial and accordingly pharmacological studies were undertaken in laboratory animals.

Procedure. Mice, rats, cats, and dogs were employed. The mice in all cases were male Swiss albino (Millerton Research Farms). Male CFW (Carworth Farms Wistar) rats were used for the toxicological studies while the pathological studies were carried out with male CFN rats (Carworth Farms Nelson) (3,4). The latter are Wistar rats which have been bred free of chronic murine pneumonia. Most of the toxicological, hematological, biochemical, and pathological methods employed herein have been described(5,6). As the 4-APP was found to be hepatotoxic, alkaline phosphatase in plasma was added to other previously described tests of liver function in dogs. The procedure of Bodansky with the modification of Gomori(7,8) was employed for this purpose. For administration of animals the agent was solubilized in H_2O by the addition of molar equivalent amounts of HCl.

TABLE I. Toxicity of 4-APP.

Species	Route of admin.	No. of successive daily doses	LD_{50}	19/20 confidence limits*	S*
			⌐mg/kg/day¬		
Mice	Intraper.	1	290	260–320	1.11
	"	5	25	18– 64	1.8
	Oral	1	180	116–279	2.4
	"	5	12.5	11– 15	1.20
Rats	Intraper.	1	228	127–410	1.54
	"	5	17.5	10– 32	2.21
	Oral	1	141	108–185	1.42
	"	5	18	15– 20	1.10
Cats	Intrav.	1	50–100	—	—
Dogs	Intrav.	10†	1.25–2.5	—	—
	Oral	10†	1.25–2.5	—	—

* Calculated according to Litchfield and Wilcoxon(9). Animals were observed for at least 2 wk after end of treatment.

† Maximum No. of doses given daily excepting weekends.

Toxicity in rodents. In Table I are presented LD_{50} data in mice and rats. 4-APP was shown to be of the same order of toxicity in both species and to be as toxic, or possibly more toxic, when given by the oral route than when injected intraperitoneally. The most prominent finding in the rodents given high single doses either by mouth or intraperitoneally was the appearance within two hours of blepharostenosis, blepharospasm and hypersensitivity. Of those given 400 mg/kg intraperitoneally all of the rats and most of the mice died unobserved overnight; the remaining mice lost weight progressively and died 12 to 13 days later. Single lethal doses given orally to mice (equal to or less than 400 mg/kg) resulted in delayed deaths between 3 and 14 days after intubation. In rats oral doses of similar magnitude caused death between 1 and 4 days. Weight loss was the only consistent finding associated with the delayed

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fatalities; an occasional animal appeared unkempt or showed diarrhea. Rats and mice which had received 4-APP on each of 5 successive days by either route of administration showed progressive weight loss beginning 24 hours after the first dose. Such animals became unkempt and occasionally the rats had "bloody" exudates about the nares. Most of the deaths occurred between the fifth and seventh days after initiation of treatment.

Pathological changes in rats. The lesions associated with intoxication in rats were studied in the CFN strain. For this purpose an initial group of 8 animals was injected intraperitoneally with 4 successive daily doses of 25 mg/kg/day. At 96 hours after initiation of this treatment the animals showed only a moderate weight loss and appeared less intoxicated than desired for the pathological studies. Accordingly they were not sacrificed and all eventually regained their lost weight and survived. (It is interesting to note that in the CFW strain this dose had proved to be greater than the LD_{50}. The lesser susceptibility of the CFN strain in this instance is in keeping with our recent experience comparing the toxicity of various agents in both kinds of Wistar rat.) A second group of CFN rats was then injected with 50 mg/kg/day for 4 days. Six of these were sacrificed at 96 hours at which time all showed severe weight loss and an untidy, unthrifty appearance. The remaining 2 rats were dead by the seventh day. The *gross findings* in all the 6 sacrificed animals were: an average weight loss of 20%, pale livers, small spleens and pallor of the renal cortex. Two showed dark purple adrenals. On microscopic examination a fat nephrosis was found in the kidneys as evidenced by slight vacuolization in the epithelium of proximal convoluted tubules. The vacuoles were primarily subnuclear and were shown to contain fine drops of fat in frozen sections stained with Sudan IV. A fine, diffuse cytoplasmic vacuolization was also seen in hepatic cells and shown to be fat by the Sudan IV stain. Except for the fatty change the livers appeared normal; there was no evidence of necrosis, inflammation or bile duct involvement. The sternal bone marrow showed an 85-90% loss of nucleated elements. In 2 animals recent cortical hemorrhages were present in the adrenals and in one of this pair the medulla was similarly involved. The adrenals of a third animal showed only medullary hemorrhages. In all 6 animals the red pulp of the spleen was reduced in amount and, though the thymus was involuted, the other lymphoid tissues showed no significant microscopic changes. No alterations were observed in the intestines or in the other viscera studied.

Toxicity in cats. Three respective pairs were given single intravenous doses of 100, 50, and 25 mg/kg. The highest dose caused immediate urination, salivation, hyperpnea and panting, a sluggish pupillary reflex, and relaxation of the nictitating membrane. At 5 minutes one of the pair given this dose had slight convulsive movements and was unable to stand. Both cats vomited several hours after injection and at 6 hours, though hyperpnea and salivation had ceased, the animals appeared moderately depressed. Thereafter depression and ataxia were evident until the animals died, one during the second day and the other during the third day. Except for retching and vomiting at 3 to 4 hours after 50 mg/kg the two lower doses produced no significant signs of intoxication and all 4 recipients survived the 3-week period of observation.

Toxicity in dogs. Each of the following lethal doses were tested in a pair of animals: 10, 5, and 2.5 mg/kg/day intravenously and 5 and 2.5 mg/kg/day by mouth. The 2 given 10 mg/kg/day died at 5 and 6 days after initiation of treatment; 7 of the remaining 8 died between 7 and 16 days; the 1 survivor had received 5 mg/kg/day by mouth. During the course of intoxication weight loss, anorexia, and vomiting appeared in all animals between the second and fifth days after initiation of treatment. Small ulcerations of the buccal mucosa were noted in 2 of the 10 dogs while 2 others showed patches of slight congestion in this area. Increases in fluid intake and in urine output with a concomitant fall in the specific gravity of urine (to 1.010 or less) were noted in 8. Nine developed loose stools by the third day and 4 of these showed tarry stools with evidence of blood by the

end of the first week. Four others had tarry stools on the day of death. Jaundice was present at 7 to 10 days (as evidenced by a yellow cast in the sclera and buccal mucosa) in 2 of the animals given 2.5 or 5 mg/kg/day intravenously. There was no jaundice seen in any of the dogs treated orally with 4-APP. Body temperatures remained normal throughout the course of fatal intoxication except for hypothermia 24 hours before death in the 2 given 10 mg/kg/day intravenously. Pairs of animals were also given doses of 1.25 mg/kg/day and 0.63 mg/kg/day, either orally or intravenously; all of these dogs survived and showed no untoward signs.

Pathological changes in dogs. Table II presents hematological and biochemical findings in a group of 4 dogs treated with intravenous doses of 4-APP. Dogs 1 and 2 received 5 mg/kg/day for 4 consecutive days while dogs 3 and 4 were given 9 successive daily injections of 2.5 mg/kg/day excepting weekends. All 4 animals were sacrificed 24 hours after the last injection. The course of intoxication in each was like that described above.

All developed reticulocytopenia and a depression of the nucleated cell count in bone marrow as seen in samples aspirated from the iliac crest. In 3 of the animals hemoconcentration was present at the time of sacrifice; in dog 4 an increased hematocrit was seen at 7 days. Significant lymphocytopenia was noted in all dogs; conversely 3 showed a neutrophilic granulocytosis. Dogs 3 and 4, which had been subjected to the more prolonged course of intoxication, had decreased numbers of circulating platelets by the eleventh day. Both dogs receiving the higher dose (5 mg/kg/day) had an obvious defect in blood coagulation as evidenced by the marked prolongations of clotting times (Table II). This disturbance was found to be associated with deficiencies of 2 of the components of the blood clotting mechanism. In Table II it will be seen that prothrombin times became greatly elevated and simultaneously the fibrinogen contents of plasma decreased significantly. Dogs 3 and 4 which had received 2.5 mg/kg/day shown no significant changes in clotting time but did develop increased pro-

TABLE II. Hematological and Biochemical Findings in Dogs Given 4-APP.

Dog	Day	Wt (kg)	Herit. (ml/100 ml)	Retic. (%)	PMN-N	Leyte	Pltlt.	BMNC	Clot time (min.)	Fbgen	Blrbn	BSP	Prot. time (sec.)	Alk. ptase (mg %)
					(10^3 cells/mm^3)					(mg %)				
1	0	12.3	57	.1	7.8	1.4	134	170	5.8	216	.3	.2	13.6	.3
	4†	10.2	77	0	22.3	.2	192	24	>22.0	68	1.2	2.8	52.1	4.7
2	0	10.7	47	.1	5.2	1.1	190	126	3.8	305	.2	.3	12.4	2.5
	4	9.2	56	0	21.6	0	164	16	22.0	*	3.9	—	>120	7.8
3	0	9.8	52	.2	14.6	1.1	432	211	4.5	—	(1)	.1	15.4	(2)
	7	7.9	61	0	10.5	.2	262	24	6.8	—	—	—	16.3	—
	11†	7.1	59	0	13.6	2.1	106	—	8.5	—	.5	1.3	35.2	2.1
4	0	15.9	47	.8	8.8	2.3	206	224	5.0	—	(1)	.2	12.6	(2)
	7	13.7	53	0	9.6	3.8	218	61	5.3	—	—	.3	12.1	—
	11	13.5	47	0	16.1	.7	34	41	6.8	—	.4	.8	18.1	3.6

Abbreviations: Alk. ptase, plasma alkaline phosphatase; Blrbn, total plasma bilirubin; BMNC, bone marrow nucleated cells; BSP, bromosulfalein retention in blood; Clot, clotting; Fbgen, plasma fibrinogen; Herit, hematocrit; Leyte, lymphocytes; Pltlt, platelets; PMN-N, polymorphonuclear neutrophils; Prot., one-stage prothrombin; Retic., reticulocytes.

* The oxalated plasma failed to clot upon addition of $CaCl_2$ and thrombin. † Dogs 1 and 3 were moribund at sacrifice.

(1) Avg control value for 49 dogs = .2 ± .1; (2) avg control value for 30 dogs = 1.3 ± .9.

thrombin times. No plasma fibrinogen determinations were done in these dogs.

Dogs 1 and 2 had significant increases in plasma bilirubin. In dogs 1, 3 and 4 in which measurements were made on the day of sacrifice bromosulfalein (BSP) retention was found to be elevated. The extent of this elevation in dogs 1 and 3 may have been exaggerated by the concomitant hemoconcentration and moribund state of these animals. Changes in plasma alkaline phosphatase were not impressive.

In additional biochemical analyses, which are not recorded in Table II, plasma protein and blood glucose remained relatively constant. Non-protein nitrogen in plasma rose from initial values of 15-25 mg % to 32-59 on the day of sacrifice. Dogs 2 and 3 which had developed hemoconcentration also showed significant decreases in plasma chloride (from 116 and 113 to 100 and 105 mEq/l respectively; unfortunately this analysis was not carried out in plasma from dog 1.

On *microscopic examination* the most consistent findings were in the livers of all 4 dogs. In dogs 1 and 2 which had been given the higher daily doses of 4-APP a fine diffuse cytoplasmic vacuolization was present in the hepatic cells; in frozen sections Sudan IV stain revealed this to be fat. In addition both livers showed plugging of the bile canaliculi; one had a slight cholangiolitic proliferation. In neither animal was there evidence of any necrotic or inflammatory changes in the liver. Other significant findings in dogs 1 and 2 were recent hemorrhages in the adrenal cortices of both and in the medulla of one. One of this pair also had hemorrhages in the myocardium, lungs, and small and large intestines. The bone marrows of both were congested but the nucleated elements appeared normal. Dogs 3 and 4 which had received the lower daily doses also had fatty livers but to a lesser extent than did 1 and 2. Again neither necrotic nor inflammatory cells were present; in this pair abnormalities were not noted in the bile ducts and canaliculi. As in dogs 1 and 2 sections of vertebral and sternal bone marrow revealed marked congestion but again the number of nucleated elements was within normal limits. No pertinent changes were seen in the kidney, intestine, spleen, or any of the other viscera studied.

Discussion. Of the purine and purine analogs which have been studied toxicologically (10) 4-APP, an isomer of adenine, is the only one known to cause fatty livers in rats and dogs although 2,6-diaminopurine has been found to induce fatty metamorphosis in pigs (11). The present study has shown that in dogs the fatty change may be accompanied by stasis of bile in the canaliculi and by evidence for hepatic insufficiency, that is, elevations in the one-stage prothrombin time and decreases in the fibrinogen content of plasma (12,13). Such changes appear in conjunction with and probably account for the blood coagulation defect. Jaundice and increased plasma bilirubin may also be seen; the hyperbilirubinemia may be related to the intrahepatic biliary stasis.

The hepatic changes produced by 4-APP can be distinguished from those caused by a close analog, 6-mercaptopurine(5,10). The latter induces hepatic necrosis in mice and rats without fatty alteration. In dogs the same agent produces stasis of bile in canaliculi similar to, but more extensive than, that caused by 4-APP; there is no evidence for fatty metamorphosis. Interestingly, the changes in tests of hepatic function in dogs intoxicated with 6-mercaptopurine are those expected to result from biliary obstruction: hyperbilirubinemia, increased retention of BSP, and marked rise in plasma alkaline phosphatase. Prothrombin time, however, remains unaltered(10).† In contrast the prominent sign of hepatic insufficiency resulting from 4-APP is the disturbance of the prothrombin complex.

Microscopic study revealed no significant change in the kidneys of dogs though fat nephrosis was observed in lethally intoxicated rats. These observations are in contrast to those previously reported with adenine, 2-chloroadenine and purine(10). Intoxication of rats with the last-mentioned purines is characterized by the deposition of occlusive

† Unpublished.

crystals in the renal tubules.

Rats given 4-APP also showed a significant decrease in the content of nucleated cells in the bone marrow. In intoxicated dogs, though bone marrow aspirated from the iliac crest contained decreased numbers of nucleated elements, microscopic study of decalcified sections revealed marked congestion in the marrow capillaries but neither decreases nor abnormalities in hematopoietic elements. It is possible that the aspirated marrow appeared depleted due to dilution with blood from the congested capillaries.

Adrenal cortical hemorrhages were found in several of the rats and dogs which had been sacrificed for pathological study. The significance of these lesions is obscure; but their presence is interesting in light of the finding that 4-APP also induces hypochloremia and polyuria in dogs.

Summary and conclusions. The toxic effects of 4-aminopyrazolo-(3,4-d)-pyrimidine (4-APP) have been studied in mice, rats, cats, and dogs. The agent was found to produce a fatty change in liver which was associated neither with necrosis nor with inflammation. Hepatic insufficiency was evidenced by hyperbilirubinemia, increases in one-stage prothrombin time and decreases in plasma fibrinogen. In rats fat nephrosis and depletion of hematopoietic elements in bone marrow were also seen. Hepatic insufficiency should be anticipated as a possible hazard in clinical trials with 4-APP.

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Demonstration of a Marked Bence-Jones Proteinemia.* (22770)

JANE I. MORTON AND H. F. DEUTSCH

Department of Physiological Chemistry, University of Wisconsin, Madison

Previous reports of Bence-Jones proteinemia have been founded upon a variety of observations: precipitate formation in multiple myeloma sera when heated to 55-60°C(1-3); aberrant serum protein solubility characteristics on salt fractionation(4); undemonstrated but postulated identity of a crystalline substance from a myeloma serum with crystals in the corresponding Bence-Jones protein containing urine(5); appearance of a new or elevated serum peak in electrophoretic pattern which closely corresponds in mobility to that of urinary Bence-Jones protein(4); immunological cross reaction with and blocking of antibody to Bence-Jones protein by the myeloma serum(6); and demonstration of slow sedimenting serum components which are electrophoretically in the γ- and β-globulin areas(6).

Most of these criteria can not be considered

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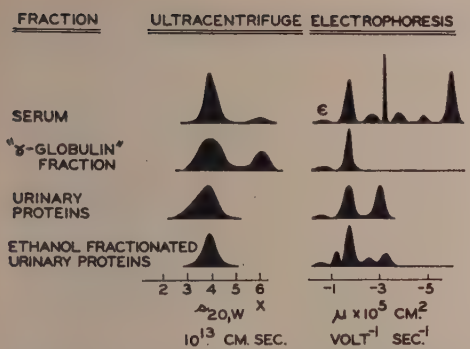


FIG. 1. Ultracentrifuge and electrophoresis diagrams of the serum and urinary proteins and fractions thereof from a case of multiple myeloma with Bence-Jones proteinemia.

in themselves adequate evidence of serum Bence-Jones protein, although the above experiments strongly suggest its presence. In this study, analysis of a multiple myeloma serum and fractions thereof by a combination of electrophoretic and ultracentrifugal technics has clearly demonstrated a considerable amount of Bence-Jones protein. This serum entity has properties in common with one of the major protein components of the urine.

Experimental and results. A patient with clinically diagnosed multiple myeloma had a total serum protein of 8.9% and an A/G ratio of 0.94 as determined by Howe fractionation. Electrophoretic analysis of the serum in pH 8.6, 0.1 ionic strength diethylbarbiturate buffer showed an increased γ-globulin area comprising 26% of total protein. The electrophoretic and ultracentrifuge diagrams of this serum and other protein systems studied, are presented in Fig. 1. The ultracentrifuge analysis of the serum carried out in the Spinco apparatus at 59,780 r.p.m. in pH 7.4, 0.2 ionic strength potassium phosphate buffer evidenced three components. These consisted of the usual small amount of an 18 S component and 2 other slower sedimenting boundaries. Only the latter 2 are seen in Fig. 1. The minor of these had an $s_{20,w}$ near 6 S and comprised 16% of the total. The slower peak had an average sedimentation constant of 3.87 S. The small amount of the 6 S component of this serum was surprising in view of the enhanced γ-globulin level (26% as compared to normal serum level of near 12%). This result suggested that the elevated γ-globulin area might also contain considerable amounts of a low molecular weight protein.

The serum was fractionated by cold ethanol technics(7) to remove the γ-globulin fraction. Electrophoretic examination of the "purified" γ-globulin fraction (see Fig. 1) revealed a single component with a mobility of -1.8×10^{-5} cm^2 $volts^{-1}$ $sec.^{-1}$ at pH 8.6. It is represented by a single symmetrical electrophoretic peak at pH values from 4.6 to 8.6. The isoelectric point was 5.9.

Ultracentrifugal analysis of this material, however, showed that 65% of it consisted of protein sedimenting with the velocity of a Bence-Jones protein (8,10). Its $s_{20,w}$ was 3.87 S and showed no concentration dependency from 0.47 to 3.0%. The faster component sedimented with a velocity of 6.02 S and thus appears to be a γ-globulin. The yield data indicated that the slower sedimenting material was present in the serum at a level near 1.4% and thus comprised near 16% of the total serum proteins.

The patient evidenced an "albuminuria" of about 10 g/day. Electrophoretic examination of the dialyzed urine and of the urinary proteins insoluble in 50% saturated ammonium sulfate revealed 2 main components. One of these components had electrophoretic mobility of a β-globulin while the other was near that of serum γ-globulin peak. Data for electrophoretic mobility of the latter urinary component and serum γ-globulin fraction as a function of pH is plotted to give Fig. 2. It is apparent that the charge relationships of the

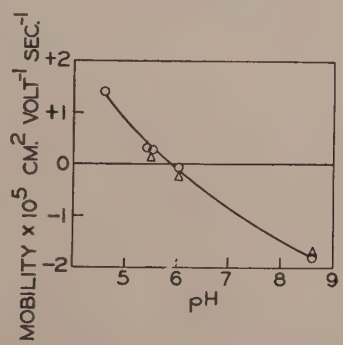


FIG. 2. Electrophoretic mobility of the serum "γ-globulin" fraction –○–○– and the major urinary component –△–△– as a function of pH.

protein from blood and urine are quite similar.

Ultracentrifuge analysis of the urinary proteins revealed a broad peak, with about 35% of the material sedimenting as a slow shoulder of the main component. The latter sedimented near 3.8 S.

The urinary proteins insoluble in 50% saturated ammonium sulfate were precipitated twice with ethanol (pH 7.0, 20% ethanol, 0.01 ionic strength, –6°C) in an attempt to purify the component with electrophoretic mobility of a γ-globulin. The resultant material when examined in the ultracentrifuge showed none of the slow shoulder characteristic of the crude urinary protein (Fig. 1). The sedimentation constant was 3.87 S, which is the same as that obtained for the low molecular weight portion of the serum γ-globulin fraction. The electrophoretic pattern (Fig. 1) shows that the minor urinary protein components had been concentrated.

Neither urinary nor serum proteins nor fractions thereof evidenced the typical Bence-Jones protein heat precipitation reaction. They coagulated on heating to near 70°C and failed to redissolve at 100°C. Filtrates of the boiled material did not give any precipitate on cooling. Nevertheless, their size and charge properties, their association with clinically diagnosed myeloma and their presence in the urine indicate that these are Bence-Jones proteins. Analogous heat reactions of such proteins have been encountered previously(9,10).

Discussion. The above results offer conclusive evidence that a protein corresponding to urinary Bence-Jones proteins may be present in certain myeloma sera. Attention should be called to the fact that a combination of fractionation, electrophoretic, and ultracentrifuge experiments on both serum and urine are required to clearly show this. Previous work using various single methods of study have not been entirely convincing. Shortcomings are present in conclusions based on electrophoretic similarity of serum components to Bence-Jones proteins without concurrent sedimentation studies being carried out. Immunological cross-reaction and blocking between the lower molecular weight Bence-Jones protein and the serum globulin components may be so strong that distinction by this means is not possible(11).

The serum protein studied here showed no concentration dependency of its sedimentation constant and would thus appear to be a relatively symmetrical molecule possessing a molecular weight of 35,000 to 40,000(9,10). The ability of this small molecule to accumulate in the serum is surprising. Previous studies in the dog have indicated that molecules of this size are cleared through the kidney quite rapidly(12). Furthermore, most myeloma patients with Bence-Jones proteinuria do not appear to show a significant Bence-Jones proteinemia(3,6).

It might be pointed out that the relatively high incidence of "albuminuria" reported in the condition of multiple myeloma is likely due to Bence-Jones proteins of the heat coagulable type described in the present and in other reports(9,10).

Summary. Simultaneous electrophoretic and ultracentrifugal studies of the serum proteins of a multiple myeloma patient have conclusively demonstrated a considerable Bence-Jones proteinemia. The properties of the serum component correspond to those of one of the major urinary components.

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Distribution and Partial Purification of Pituitary Gonadotropins of Human Plasma.* (22771)

J. W. McArthur, R. B. Pennell, H. N. Antoniades, F. M. Ingersoll, J. L. Oncley, and H. Ulfelder (Introduced by Joseph C. Aub)

Vincent Memorial Hospital, Protein Foundation, Inc. and Departments of Gynecology and Biophysical Chemistry, Harvard Medical School

The presence of a substance or substances possessing gonadotropic activity, presumably of pituitary origin, in the serum of ovariectomized women was first demonstrated by Fluhmann(1). Subsequently, Cohn and his collaborators(2) reported the detection of two gonadotropic hormones in fractions prepared from certain pools of human plasma. Since these findings could not be fully confirmed in later studies,† we have reexamined the distribution of pituitary gonadotropic activity among various plasma fractions. The bulk of the gonadotropic activity has been located in Fraction II + III, and by subfractionation has been partially purified. Preliminary reports of these studies have appeared elsewhere (3,4).

Methods and materials. Postmenopausal plasma was employed exclusively in this study, preliminary bioassays having shown that gonadotropic activity is readily demonstrable in the whole plasma of postmenopausal women, but not in that of men or of adult women of menstrual age. The donors were healthy women 65 years of age or less who were not undergoing treatment with estrogenic preparations of any kind.‡ The blood was collected either with citrate or through cationic exchange resins in the ADL-Cohn blood fractionating machine. The data herein reported were obtained by fractionating a 10-liter plasma pool which comprised the donations of 40 women, and are identical with the results previously obtained from the fractionation of 20 individual or pooled plasma samples from 3-12 donors. The cold ethanol method VI described by Cohn *et al.* (6) was employed for the initial plasma fractionation. The following fractions were prepared: Fraction I containing the fibrinogen; II + III consisting of β-lipoproteins and gamma globulin; IV-1 containing the α-globulins and cholesterol; IV-4 consisting of α- and β-globulins and the metal-combining globulin; V, which contains the bulk of the albumin; and VI, the supernatant remaining after fractionation. Partial purification of the gonadotropically-active material in Fraction II + III was accomplished by precipitating the bulk of the biologically-inactive proteins with zinc. Fraction II + III was brought to the original plasma volume with 0.15 M sodium chloride and zinc glycinate added until a 5 mM zinc concentration was obtained at a pH of 6.8 and a temperature of 0°C. The supernatant was freed of zinc by dialysis against ethylene diamine tetra-acetic acid (EDTA) and sodium citrate. The EDTA and citrate were subsequently removed by dialysis against 0.15 M sodium chloride. The addition of 20% ethanol to the purified supernatant at a pH of 6.5 and a temperature of —5°C resulted in the precipitation of additional material which was biologically inactive, leaving the gonadotropins in a supernatant containing 2-4% of the proteins originally present in the fraction. In preparation for bioassay the fractions were redissolved in small volumes of 0.15 M NaCl and dialyzed for 24 hours against 0.15 M NaCl in order to remove residual alcohol and excess salt. All samples were stored at 2°C and their pH adjusted to 7.0 ± 0.4 immedi-

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† Unpublished data.

‡ Previous experience has shown that unwitting ingestion of estrogen occasionally results in significant contamination of plasma pools. Therefore, the whole plasma and those fractions found to produce an increase in uterine weight of intact rats were assayed for estrogen in spayed rats by modification of method of Bulbring and Burn(5). No indication of estrogenic activity was found.

TABLE I. Relative Gonadotropic Activity of Whole Postmenopausal Plasma and of Its Individual Fractions.

Preparation	% plasma proteins	Plasma equivalence (ml)	No. of animals	Mean wt, mg* Uterus	Ovary
Whole plasma	100.0	15	3	24.6	17.2
		20	3	*91.5*	14.0
		40	6	*110.1*	*24.6*
I	4.1	400	3	15.2	11.3
II + III	25.0	15	2	23.3	14.8
		20	3	*39.7*	16.7
		40	3	*97.3*	16.1
		80	3	*131.4*	*21.8*
IV-1	7.5	400	3	23.9	15.9
		500	3	*58.6*	12.1
		666	3	*146.5*	15.7
IV-4	11.6	550	3	26.3	14.7
		700	3	*125.5*	18.8
V	50.0	600	3	24.9	18.9
		750	2	*73.0*	18.5
VI	1.0	700	3	33.2	16.9

* Mean uterine weights in excess of 38.3 and mean ovarian weights in excess of 21.1 mg are regarded as differing significantly from control weights ($p < 0.01$) and are italicized.

ately prior to assay. "Gonadotropic activity," which presumably comprises the action of two substances, pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH), was assayed biologically. Immature female rats of the Sprague-Dawley strain, weighing 45-55 g, were injected subcutaneously with the material to be tested twice daily for 4 days and were killed on the fifth day. The wet weights of the ovaries and the fluid-free uterus were employed as indices of gonadotropic activity, the minimum quantity of whole plasma and of the individual fractions required to induce statistically significant increases in the weights of these organs being ascertained. In 23 control animals, the mean ovarian weight was 15.1 ± 3.7 and the mean uterine weight 28.2 ± 6.2 mg. Therefore, that volume of plasma which just sufficed to increase the mean ovarian weight of the three test animals above 21.1 or the mean uterine weight above 38.3 mg was regarded as the minimal effective dose ($p<0.01$).

Results. The relative gonadotropic activity of whole plasma and of its various fractions is shown in Table I. The gonadotropic potency of whole plasma ($M.E.D._{ut.\ wt.}$, 20 ml; $M.E.D._{ov.\ wt.}$, 40 ml) accords well with Fluhmann's observations(1). Employing as his test animal the immature female mouse, which is approximately 6 times as sensitive to human menopausal gonadotropin as the rat (7), Fluhmann demonstrated gonadotropic activity in 3-5 ml of the serum of ovariectomized women. The finding that appreciably greater gonadotropic stimulation is required for ovarian growth than for the induction of ovarian secretory activity is in agreement with many previous observations.

The bulk of the gonadotropic activity is seen to be concentrated in Fraction II + III, although traces of activity are demonstrable in Fractions IV-1, IV-4 and V. The potency of Fraction II + III would appear to be somewhat more than half that of whole

TABLE II. Relative Gonadotropic Activity of Fraction II + III, and of Its Subfractions.

Preparation	% proteins in Fraction II + III	Plasma equivalence (ml)	No. of animals	Mean uterine wt (mg)*	Mean ovarian wt (mg)*
II + III	100	20	3	*39.7*	16.7
		40	3	*97.3*	16.1
		80	3	*131.4*	*21.8*
5 mM Zn^{++} precipitate	94	300	3	35.7	14.1
" supernatant	6	20	3	26.3	18.2
		40	3	*126.1*	18.1
		80	3	*141.9*	*35.8*
20% ethanol precipitate from 5 mM Zn^{++} supernatant	3	100	3	25.8	14.7
20% ethanol supernatant from 5 mM Zn^{++} supernatant	3	40	2	*98.8*	15.4
		80	3	*115.7*	*34.9*

* Mean uterine weights in excess of 38.3 and mean ovarian weights in excess of 21.1 mg are regarded as differing significantly from control weights ($p < 0.01$) and are italicized.

plasma, whether gauged by the $M.E.D._{ut.\ wt.}$ or the $M.E.D._{ov.wt.}$ Fractions I and VI appear to be devoid of gonadotropic activity.

The relative gonadotropic potency of Fraction II + III and of its subfractions is shown in Table II. It will be noted that zinc precipitation effectively frees the gonadotropically-active material from the majority of the contaminating proteins and that re-precipitation with 20% ethanol removes additional contaminants.

Summary. The location and approximate concentration of gonadotropic activity in various fractions of human postmenopausal plasma has been determined. The plasma was fractionated by means of the cold ethanol method VI of Cohn and was assayed in immature female rats by estimating ovarian growth and secretory activity. Although traces of activity were found in Fractions IV-1, IV-4 and V, the bulk of the gonadotropically-active material was located in Fraction II + III. Subfractionation of Fraction II + III by successive precipitations with zinc and 20% ethanol freed the gonadotropins from the majority of the contaminating proteins.

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